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The Endosomal Protein Appl1 Mediates Akt Substrate Specificity and Cell Survival in Vertebrate Development

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SUMMARY

During development of multicellular organisms, cells respond to extracellular cues through nonlinear signal transduction cascades whose principal components have been identified. Nevertheless, the molecular mechanisms underlying specificity of cellular responses remain poorly understood. Spatial distribution of signaling proteins may contribute to signaling specificity. Here, we tested this hypothesis by investigating the role of the Rab5 effector Appl1, an endosomal protein that interacts with transmembrane receptors and Akt. We show that in zebrafish, Appl1 regulates Akt activity and substrate specificity, controlling GSK-3β but not TSC2. Consistent with this pattern, Appl1 is selectively required for cell survival, most critically in highly expressing tissues. Remarkably, Appl1 function requires its endosomal localization. Indeed, Akt and GSK-3β, but not TSC2, dynamically associate with Appl1 endosomes upon growth factor stimulation. We propose that partitioning of Akt and selected effectors onto endosomal compartments represents a key mechanism contributing to the specificity of signal transduction in vertebrate development.

INTRODUCTION

Transmission of signals from the plasma membrane through cytoplasmic cascades of protein kinases is a central concept to explain how cells can regulate basic functions in response to extracellular cues. Information on the molecular players of signal transduction pathways fitting this concept has exponentially grown in the past decade, uncovering central signaling hubs such protein kinase B (Akt) and mitogen-activated protein kinases (MAPK) (Dhillon et al., 2007; Manning and Cantley, 2007). However, our understanding of the mechanisms underlying the specificity of signal transmission and processing requires new conceptual advances. Signaling components such as kinases often possess various potential substrates, leading to highly branched signaling networks rather than linear cascades. This poses the problem of how a physiological response can be elicited with high specificity, with information flowing through selected signaling components while excluding others.

Various mechanisms can modulate the activity of the signal transduction constituents, e.g., via posttranslational modifications, conformational changes, cell/tissue-specific expression, or interactions with adaptor proteins (Bardwell, 2006; Dumont et al., 2001; Hoeller et al., 2005; Pawson and Scott, 2005; Polak and Hall, 2006; Weston and Davis, 2001). On the other hand, specificity of the signaling response can also exploit spatial information and temporal dynamics (Kholodenko, 2003). A cellular process that could ideally serve both mechanisms is endocytosis (Hoeller et al., 2005; Le Roy and Wrana, 2005; Miaczynska et al., 2004b). Upon ligand binding and signal initiation at the plasma membrane, signaling receptors are internalized and transported through a series of endosomes. Endocytosis not only enables signal termination by targeting these complexes to lysosomes for degradation but also interactions with downstream signaling partners. Trafficking of TGFβ and EGF receptors, for example, permits receptor signaling by associating with the compartment-specific adaptor proteins SARA and p14 in early and late endosomes, respectively (Di Guglielmo et al., 2003; Panopoulou et al., 2002; Teis et al., 2002). However, whether the endocytic machinery not only permits signaling but also confers signaling specificity in vivo is unknown at present.

A dual function of endocytosis in trafficking and signaling is also reflected by the molecular composition of the effector machinery downstream of the small GTPase Rab5. It is well

established that Rab5 and its effectors play multiple roles at early stages of the endocytic pathway, regulating cargo sorting, early endosome fusion, actin- and microtubule-dependent motility (Hoepfner et al., 2005; Pal et al., 2006; Pelkmans et al., 2004; Rink et al., 2005). Some Rab5 effectors acting in these processes are well known signaling components regulating phosphoinositide synthesis and turnover (Christoforidis et al., 1999; Shin et al., 2005). Others play a less established, but equally important role in signaling. Two homologous Rab5 effectors, APPL1 and APPL2 (adaptor protein containing pH domain, PTB domain, and Leucine zipper motif, also termed DIP13α and β), which are associated with a subset of Rab5-positive early endosomes (Miaczynska et al., 2004a), bind to various transmembrane receptors (TrkA [Lin et al., 2006; Varsano et al., 2006], DCC [Liu et al., 2002], Adiponectin [Mao et al., 2006], FSH [Nechamen et al., 2004], and NMDA receptors [Husi et al., 2000]). Furthermore, APPL1 has been reported to interact with, and regulate the activity of, the kinase Akt (Lin et al., 2006; Mao et al., 2006; Varsano et al., 2006; Yang et al., 2003). Akt orchestrates diverse fundamental processes such as survival, growth, proliferation, and metabolism (Brazil et al., 2004; Manning and Cantley, 2007). Although Akt signaling is commonly believed to initiate at the plasma membrane, it also depends on receptor endocytosis (Hunker et al., 2006; Su et al., 2006). The mechanisms and physiological relevance of endocytosis for Akt signaling are at present entirely unknown.

Here, we took advantage of the zebrafish *Danio rerio* as model organism to explore the function of APPL proteins and determine whether signaling from an endosomal compartment results in a specific biological response in vivo.

RESULTS

APPL Proteins Are Highly Conserved in Vertebrates

APPL1/2 homologous sequences were found in all sequenced vertebrate genomes including that of the zebrafish *Danio rerio*. Starting from annotated sequence fragments, we cloned their coding regions and 5′-UTRs by RACE-PCR. Zebrafish Appl proteins harbor the same functional domains as their human counterparts (Figure 1A) and show an overall conservation of 80% and 65%, respectively (Figure S1 available online).

Zebrafish Appl1 and Appl2 Are Rab5 Effector Proteins and Display Endosomal Localization

We tested whether also zebrafish Appl proteins exhibit properties of Rab5 effectors (Miaczynska et al., 2004a). First, we coexpressed fluorescently tagged CFP-Rab5C and Appl1/2-Venus proteins in the developing fish. In vitro transcribed *appl1* or *appl2* mRNAs were injected at 1 cell stage, giving rise to ubiquitous expression, whereas *rab5C* mRNA was injected in single blastomeres at 16 cell stage, to generate mosaic expression. In coexpressing cells, Appl1- and Appl2-Venus colocalized with CFP-Rab5C in a characteristic endosomal pattern (Figure 1B, arrows; Figure S2). Moreover, coexpression of CFP-Rab5C gave rise to larger and brighter Appl1- and Appl2-Venus structures (Figures 1C and S2), indicating enhanced recruitment of Appl1 and Appl2 by Rab5.

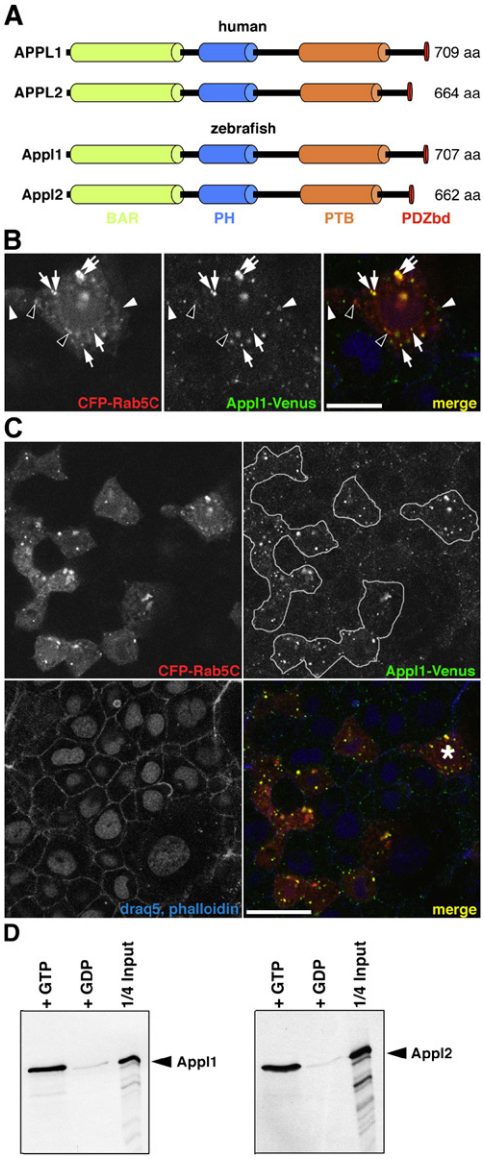

Figure 1. Appl Proteins, Their Localization, and Biochemical Properties Are Conserved in Zebrafish

(A) Schematic representation of human and zebrafish APPL proteins indicating functional domains and total length.

(B and C) Zebrafish embryos injected with 100 pg *appl1-Venus* mRNA at single-cell stage and with 50 pg *CFP-Rab5C* mRNA into single blastomeres at 16-cell stage, fixed, and imaged at 50% epiboly stage. Single confocal sections are shown. Draq5 and phalloidin staining highlight nuclei and cell margins in blue.

(B) A cell coexpressing fluorescently labeled Appl1 and Rab5C proteins. Many labeled structures are shared between the two proteins (yellow, in merged panel, few highlighted by arrows). White arrowheads, structures that carry only Appl1-Venus; empty arrowheads, structures labeled by Rab5C only. The scale bar represents 10 μm.

(C) Distribution of ubiquitously expressed Appl1-Venus is altered in CFP-Rab5C coexpressing cells (outlined by white line): bigger and brighter structures are observed. Asterisk indicates cell represented in (B). The scale bar represents 20 μm.

(D) GST pull-down assays. Recombinant GST-Rab5C protein, loaded with either GTPγS (+GTP) or GDP (+GDP) nucleotides. In vitro translated Appl1 and Appl2 proteins bind specifically to Rab5C+GTP.

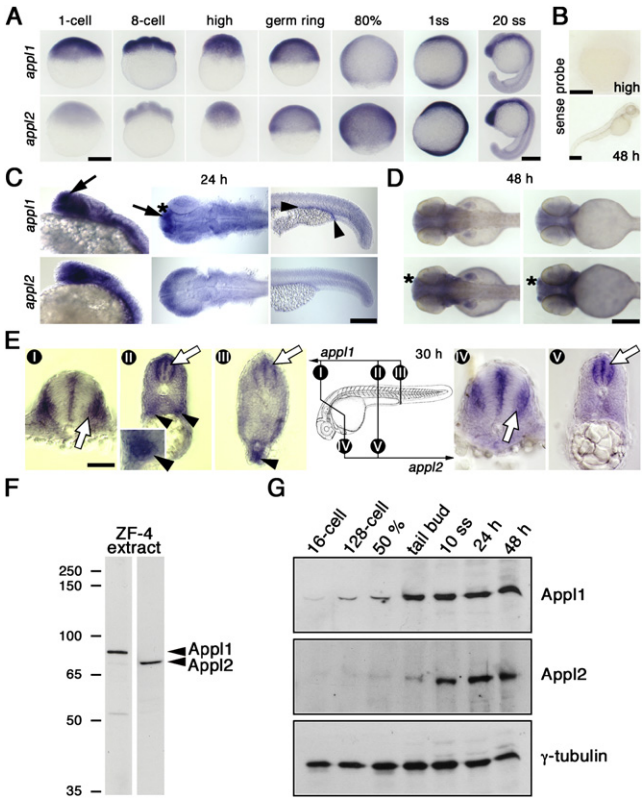

Figure 2. *appl* Developmental Expression Profiles

(A) *appl1* and *2* mRNA patterns during early embryogenesis, revealed by antisense probes. Lateral views. %, % epiboly; ss, somites. Both genes are ubiquitously expressed.

(B) Simultaneous ISH with an *appl1* sense probe. No unspecific background staining was observed.

(C) *appl1* and *2* mRNA expression at 24 hr of zebrafish development. Left panels, heads in lateral view; mid panels, heads from top; right panels, tails in lateral view. High expression of *appl1* is observed in telencephalon (arrows), olfactory pits (asterisks), and pronephros (arrowheads).

(D) *appl1* and *2* mRNA expression at 48 hr of zebrafish development. Left panels, dorsal views; right panels, ventral views on the zebrafish trunk. *appl2* mRNA is enriched in the olfactory placode (asterisks).

(E) Sections through *appl1* (panels I–III) or *appl2* (panels IV and V) whole-mount ISH of 30 hr zebrafish. Positions of sections are indicated in the scheme. Empty arrows, high expression of *appl1* and *appl2* in specific zones of the hindbrain (I and IV) and neural tube (II, III, and V). Arrowheads, *appl1* in the pronephric duct.

(A–E) *appl1* and *2* whole-mount ISH (in violet) on zebrafish embryos of indicated developmental stages. The scale bars in (A) and (B) represent 250 μm; in (C) and (D), 200 μm; and in (E), 50 μm.

(F) Antibodies to Appl1 and Appl2 recognize single bands of 80 and 75 kD in immunoblots on ZF4 extract.

(G) Western blot analysis of staged zebrafish embryos using anti-Appl antibodies. Anti-γ-tubulin was used as loading control.

Second, the ability of fish Appl proteins to bind Rab5 was tested in a GST pull-down assay using recombinant GST-Rab5C preloaded with either GTPγS or GDP and in vitro translated Appl1 and Appl2. They strongly and specifically bound to the active form of Rab5C (Figure 1D).

Therefore, Appl proteins are endosomal effectors of Rab5 in fish, implying their functional conservation in vertebrate evolution.

Appl Proteins Are Expressed Early, Widely, and Enriched in Forebrain, Pronephros, and Neural Tube during Embryogenesis

To determine the developmental expression pattern of APPL proteins, we carried out whole-mount in situ hybridization (ISH) on zebrafish embryos. We found that *appl1* and *appl2* are widely expressed during early embryogenesis and maternally provided, their mRNAs being detected before onset of zygotic gene expression (see Figure 2A, 1- and 8-cell stages; Figure 2B). From embryonic day 1 on, *appl* expression remains ubiquitous, but is elevated in certain tissues, such as telencephalon and pronephros (*appl1*, Figures 2C and 2E) as well as in the olfactory organ and neural tube (*appl1* and *appl2,* Figures 2C–2E). The *appl1* and *appl2* expression patterns are thus overlapping, but not identical.

To detect expression at the protein level, we raised antibodies against the Appl proteins. By western blot analysis on extracts of the fish cell line ZF4, these antibodies revealed single bands of the predicted molecular weight (Figure 2F). The western blot profile of fish extracts from different developmental stages (Figure 2G) and the protein pattern revealed by whole-mount immunostaining (Figures 3A–3B″) entirely correlate with the ISH data. Labeling was specific, as immunoreactivity was strongly reduced in fish injected with antisense morpholinos (MOs) preventing Appl1 translation (Figures 3C and 4A).

Appl Proteins Label Noncanonical Early Endosomes throughout Development

We next examined the subcellular localization of the endogenous proteins. Whereas Appl2 was undetectable during early embryogenesis (data not shown), consistent with western blot analysis (Figure 2G), anti-Appl1 antibodies revealed a characteristic endosomal pattern underneath the plasma membrane (Figures 3D–3D″) whereas EEA1, a characteristic marker of early endosomes, was scattered throughout the cytoplasm (Figures 3E–3E″). These two features, enrichment in the cell periphery and segregation from canonical early endosomes, are hallmarks of mammalian APPL endosomes (Miaczynska et al., 2004a). Specific endosomal patterns were also visible at later developmental stages (Figures 3F and 3G and data not shown). Localization of Appl1 to membrane structures was also demonstrated by cryo-immunogold-electron microscopy of fish embryos (Figure 3H). All together, our data suggest that not only Appl proteins, but also their biochemical properties, intracellular localization and Appl endosome features are conserved in zebrafish.

Appl Knockdown Causes Apoptosis

To address the physiological role of Appl proteins, we designed and tested different MOs to specifically ablate the expression of either *appl1* or *appl2*. We identified two MOs that knocked-down Appl1 (MO1A and MO1B) and one ablating Appl2 (MO2) (Figure 4A). By titrating MO doses, we found that animals injected with a "high dose" of either MO1A or MO1B (12 ng) or MO2 (4 ng), showed rudimentary yolk extensions, an overall sick appearance (Figure 4B), as well as edema and bent body axes later in development (data not shown). Staining of these animals with Acridine Orange, a vital dye that specifically labels

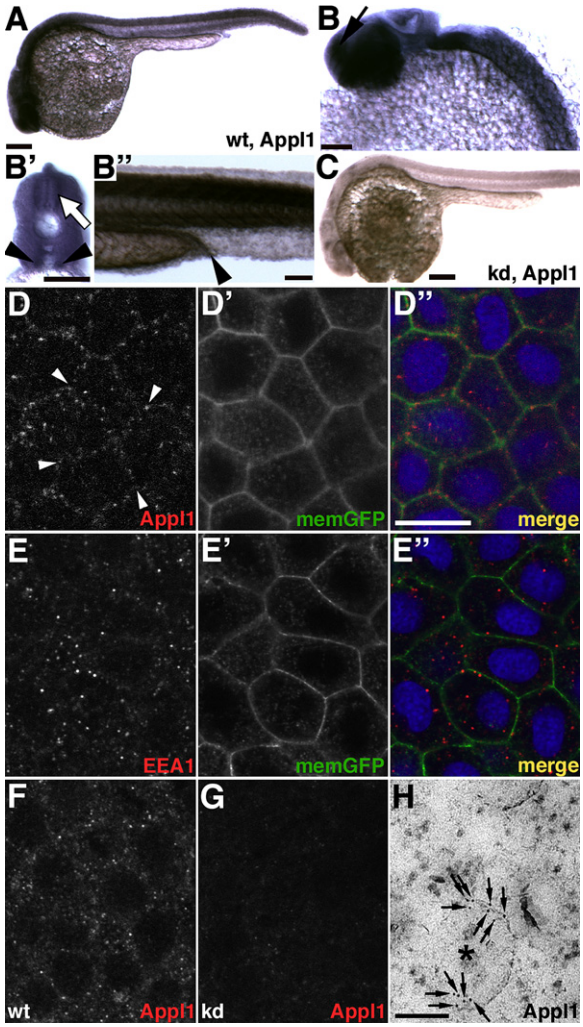

Figure 3. Appl1 Protein and Endosomes during Development

(A) Appl1 protein in a WT zebrafish.

(B) Magnification of a head in lateral view. Arrow, telencephalon.

(B′) Section at the level of the yolk extension. High Appl1 expression in the neural tube (empty arrow) and in the pronephric duct (arrowheads).

(B″) Strong pronephric expression of Appl1, (arrowhead,), lateral view.

(C) Simultaneously performed anti-Appl1 labeling on embryos injected with 10 ng *appl1* antisense morpholino. Strongly reduced Appl1 signal provides evidence for specificity of the labeling.

(A–C) Anti-Appl1 whole-mount immunohistochemistry on 26 hr zebrafish. The scale bars represent in (A) and (C), 200 μm; in (B), 100 μm; and in (B′) and (B″), 50 μm.

(D–E″) Immunohistochemistry on zebrafish embryos at 50% epiboly. Immunolabelings have been carried out on a transgenic membrane-GFP (memGFP) line (Cooper et al., 2005) to highlight cell margins.

(D) Anti-Appl1 immunolabeling, (D′) memGFP, (D″) merged image of (D) (in red) and (D′) (in green), nuclear draq is shown in blue. Note that Appl1 endosomes are enriched near cell margins (arrowheads).

(E) Immunolabeling using anti-EEA1, a marker labeling canonical early endosomes, (E′) memGFP, (E″) merged image of (E) (in red) and (E′) (in green), nuclear draq5 staining is shown in blue. EEA1 positive endosomes appear randomly scattered throughout cells.

(F) Anti-Appl1 immunolabeling on brain rudiment during early somite stages.

(G) Strongly reduced Anti-Appl1 immunolabeling on brain rudiment upon injection of 10 ng *appl1* antisense morpholino.

apoptotic cells (Abrams et al., 1993; Nowak et al., 2005) revealed strong and widely induced apoptosis (Figure 4C). Most high-dose morphants died between 3 and 6 days of development.

Fish injected with a "low dose" (MO1A or MO1B [8 ng] or MO2 [2 ng]) survived, developed normally, and were morphologically indistinguishable from their control siblings (data not shown and Figure 4D). They exhibited (1) overall normal growth and patterning, as judged by morphological inspection and ISH with a panel of early patterning markers (Supplemental Experimental Procedures) and (2) no striking defects in proliferation, as revealed by P-Histone H3 immunostaining (Figure S3). However, compared to normally occurring developmental cell death ([Cole and Ross, 2001], MOCK panels), these animals also showed a greater degree of apoptosis, except that this was restricted to those tissues exhibiting high levels of Appl1 and Appl2 expression, i.e., telencephalon, olfactory bulb, and pronephros for *appl1* (Figure 4D), and neural tube for *appl2* (Figure 4E).

Despite the aforementioned correlation, apoptosis could be interpreted as a generalized, unspecific phenotype. Therefore, to corroborate the specificity of the observed phenotypes we conducted a series of control experiments. First, coinjection of *appl1-5′ UTR*-binding MO1A along with in vitro transcribed *appl1* mRNA lacking the MO binding site rescued forebrain and pronephros apoptosis in the vast majority of embryos (Figure 4F), as assessed quantitatively in a forebrain apoptosis assay (Figure 4G).

Second, since some MOs have been recently shown to unspecifically induce p53 activity and cell death (Robu et al., 2007), we further verified the specificity of the phenotype induced by Appl1 knockdown by coinjecting a previously characterized p53 MO (Nowak et al., 2005), which reduces MO off-target effects (Robu et al., 2007). Concurrent p53 knockdown did not significantly alter forebrain apoptosis induced by Appl1 knockdown (Figure S4).

Finally, to obtain MO-independent evidence for a role of Appl proteins in cell survival, we targeted the *appl* genes by TILLING (McCallum et al., 2000). We obtained one interesting mutation in the Appl*2* protein (A196T), targeting an alanine residue within the BAR domain strictly conserved in all APPL proteins from various species (Figure S5A). Despite the presence of mutant Appl2 protein (data not shown), a subset of animals with maternal and zygotically mutated protein (from homozygous mutant in-crosses) displayed morphological phenotypes similar to those observed upon MO injection, i.e., reduced yolk extension, swollen telencephalon and edema. Importantly, they were characterized by cell death in the olfactory organ and neural tube (18%, n = 1160, from seven independent clutches) (Figure 4H). Since no phenotype was observed upon mating of heterozygous mutants, the A196T mutation likely represents a hypomorphic Appl2 allele that causes apoptosis only upon depletion of the wild-type (WT) protein.

On the basis of five independent lines of evidence, (1) the consistent phenotypes caused by different morpholinos, (2) the

(D–G) Single confocal sections are shown. Experiments were performed under the same conditions and microscope settings. The scale bar in (D″) represents 15 μm.

(H) High magnification of an anti-Appl1 immunoelectron micrograph of a 80% epiboly embryo cryosection. A 200 nm vesicle (asterisk) is decorated with anti-Appl1-directed 10 nm gold particles (arrows). The scale bar represents 100 nm.

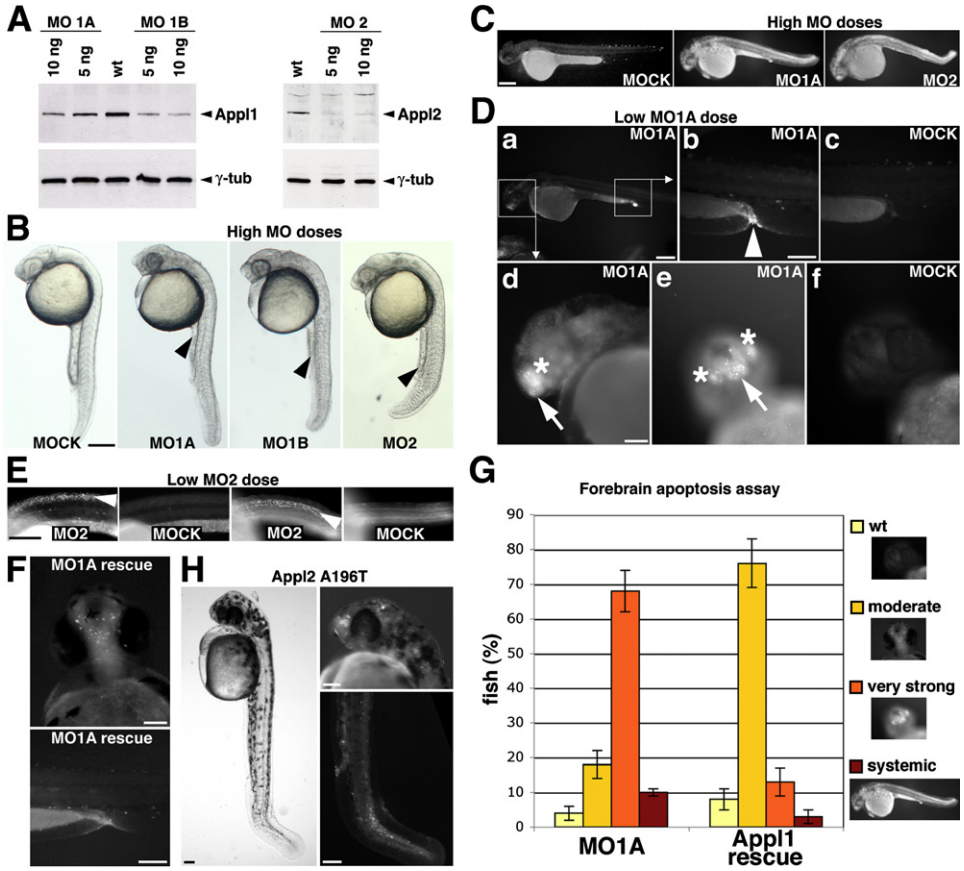

Figure 4. Loss of Appls Causes Apoptosis

(A) Reduction of Appl1 and Appl2 protein levels upon injection of 5 and 10 ng antisense MOs into single-cell stage embryos. Proteins were extracted from 10-somite embryos to illustrate dose-dependent MO-mediated knockdown efficiency. Knockdown efficiency also depends on the stage of extraction (see later extracts, Figure 5A). Two MOs knock down Appl1 (MO1A and MO1B), and one MO knocks down Appl2 (MO2).

(B) Morphological phenotypes of 24 hr zebrafish injected with either injection buffer only (MOCK) or "high" doses of MOs: MO1A (12 ng), MO1B (12 ng), or MO2 (4 ng): reduced yolk extensions and overall sick appearance. The scale bar represents 250 μm.

(C–H) Apoptotic staining by AO (in white) on MOCK-injected, morphant and TILLING zebrafish embryos at 54 hr of development. AO unspecifically accumulates in the yolk.

(C) Wide apoptosis in "high"-dose morphants (MO1A [12 ng] and MO2 [4 ng]). The scale bar represents 250 μm.

(D) Local apoptosis in "low"-dose MO1A morphants (8 ng). (a) Low-dose MO1A embryo, lateral view. Head region magnified in panels (d) and (e) and tails magnified in panel (b) are indicated. (b) Strong apoptosis of the distal pronephric tube. (c) Corresponding region of a MOCK-injected embryo. (d and e) Magnified MO1A head region, lateral (d) and front view (e). Strong apoptosis is observed in forebrain including telencephalon and olfactory placode. (f) Corresponding region of a MOCK-injected embryo (front view). The scale bars represent (a) 250 μm, (b–f) 100 μm.

(E) Local apoptosis in low-dose MO2 morphants (2 ng) is restricted to the neural tube, shown at the level of the yolk extension. Panels to the left, lateral view; panels to the right, top view. The scale bar represents 250 μm.

(F) Rescue of apoptosis in forebrain and pronephros in low-dose MO1A morphants (8 ng) by coinjection of 75 pg *appl1* mRNA that lacks the MO1A binding site. The scale bars represent 100 μm.

(G) Forebrain apoptosis assay: Quantitative evaluation of apoptosis in Appl1 knockdown (MO1A: 8 ng MO1A) and rescued zebrafish embryos (Appl1 rescue: 8 ng MO1A + 75 pg *appl1* mRNA). Embryos were assigned to one of four cell death categories: WT, moderate, very strong forebrain, and systemic apoptosis (representative images in legend). Total amount of embryos scored: $n^{MO1A} = 532$; $n^{Appl1\ rescue} = 598$. Error bars indicate SD between experiments. Note that apoptosis is significantly rescued in the majority of embryos.

(H) APPL2A196T mutant fish isolated by TILLING are characterized by reduced yolk extension, kinked tails, apoptosis in the neural tube, and olfactory placodes, resembling *appl2* morphants (left panel). The scale bars represent 100 μm.

correlation between pattern of apoptosis and pattern of high Appl expression, (3) the rescue of the apoptotic phenotype, (4) the resistance of apoptosis to concurrent p53 knockdown, and (5) the consistency of phenotypes in the genetic mutant, we conclude that Appl proteins are required for cell survival during development.

Apoptosis in Appl1 Ablated Animals Is Due to Specific Alterations in Akt Signaling

What is the molecular basis underlying Appl1-mediated cell survival? The reported interaction with, and modulation of Akt activity by, APPL1 in mammalian cells (Lin et al., 2006; Mao et al., 2006; Mitsuuchi et al., 1999; Varsano et al., 2006; Yang et al.,

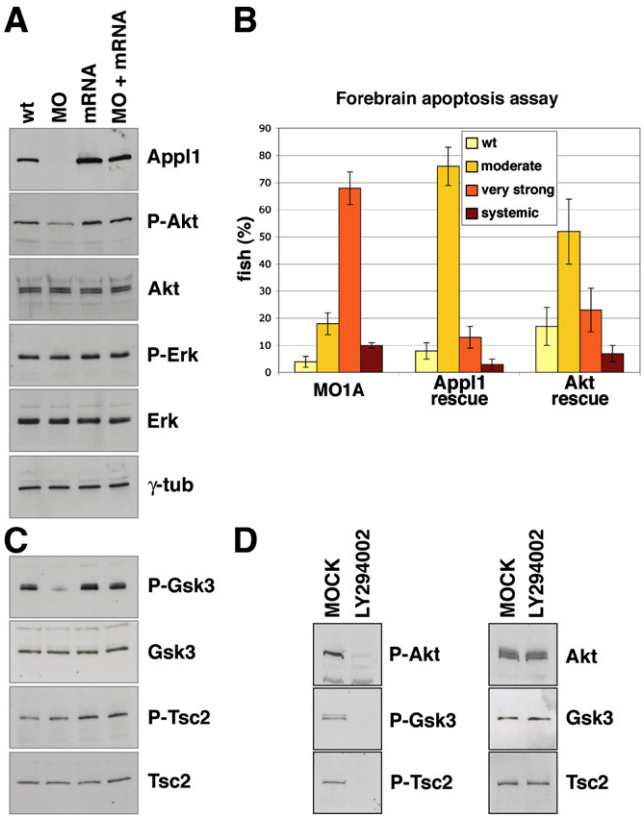

Figure 5. Appl1 Regulates Akt Activity, Akt Substrate Specificity, and Akt-Dependent Survival in Zebrafish Development

(A) Western blot analyses of Akt and MAPK level and activity in WT, Appl1 knockdown (MO: 8 ng MO1A), Appl1 overexpressing (mRNA: 75 pg *appl* mRNA), and rescued embryos (MO + mRNA: 8 ng MO1A + 75 pg *appl* mRNA).

(B) Forebrain apoptosis assay: Quantitative evaluation of apoptosis in Appl1 knockdown embryos rescued by expression of Akt (Akt rescue: 8 ng MO1A + 100 pg *Akt2* mRNA). Embryos were assigned to one of the four indicated cell death categories. Evaluation of Appl1 knockdown and *appl1* mRNA-rescued embryos (Figure 4G) are shown for comparison. Total amount of embryos scored: $n^{\text{Akt rescue}} = 396$. Error bars indicate standard deviation (SD) amongst five experiments.

(C) Western blot analyses on the same fish extracts analyzed in panel (A) monitoring level and activity of Gsk-3β and Tsc2.

(D) Western blot analyses of MOCK and LY294002-treated fish. (Ser9)-Gsk-3β and (Thr1462) phosphorylation critically depends on Akt activity.

(A–D) Experiments have been carried out on 54 hr zebrafish embryos.

2003), suggested that loss of APPL may corrupt growth factor signaling during development. We thus analyzed key growth factor-induced signaling pathways using extracts from WT, Appl1 knockdown (MO), Appl1 overexpressing (mRNA), and rescued (MO + mRNA) fish (Figure 5A). Whereas total levels of fish Akt were unaffected, endogenous Akt activity, revealed by an anti-phospho-Akt (Ser473, anti-P-Akt) antibody, was strongly diminished upon depletion of Appl1. Under these conditions, overexpression of Appl1 only mildly enhanced Akt activity. Importantly, in fish injected with both *appl1* MO and mRNA, Akt activity was restored, consistent with the rescue of cell survival (Figure 4).

In contrast to diminished Akt activity, the activity of the MAPK pathways, monitored with anti-P-ERK (Figure 5A) and anti-P-p38 antibodies (data not shown) was unaffected. This is in agreement with the recent report by Mao et al. (2006) but is in contrast to others showing no discrimination between regulation of Akt and MAPK signaling by APPL in cultured cells (Lin et al., 2006; Varsano et al., 2006; Yang et al., 2003). These results suggest that, in the context of a multicellular organism, Appl1 functions as a membrane adaptor specifically required for Akt activity and cell survival.

Is diminished Akt activity the primary cause of apoptosis induced by Appl deficiency or merely an indicator of cell death? We addressed this question by determining whether overexpression of Akt could rescue apoptosis induced upon loss of Appl1. A dose of in vitro transcribed zebrafish *akt2* mRNA that by itself does not affect cell survival was coinjected with 8 ng MO1A, and indeed rescued the apoptotic phenotype almost as efficiently as Appl1 itself (Figure 5B). We conclude that Appl1 selectively regulates apoptosis during development by controlling Akt signaling.

Appl1 Regulates Akt Signaling Specificity

The reduced Akt activity raised the question of why cell survival but not other functions under Akt control, such as growth and proliferation, are affected by loss of Appl1. This prompted us to inspect the activity of Akt substrates. We could monitor the activity of two downstream effectors of Akt, Tsc2 involved in growth control (Manning and Cantley, 2007) and Gsk-3β implicated in several processes including cell survival (Jope and Johnson, 2004; Manning and Cantley, 2007). Strikingly, whereas total levels of both proteins did not vary, their activity was affected very differently by loss of Appl1 (Figure 5C). Only residual levels of P-Ser9-Gsk-3β were detected demonstrating that its regulation heavily relies on Appl1. In sharp contrast, Tsc2 phosphorylation at Akt target site Thr1462 was unaffected suggesting that this pathway operates in an Appl1-independent manner (Figure 5C). However, exposure of fish to two pharmacological inhibitors of PI3-K, LY294002 (Figure 5D) or Wortmannin (data not shown), blocked Akt activation and abolished both Tsc2 and Gsk-3β phosphorylation, verifying that Tsc2 Thr1462 phosphorylation crucially depends on Akt in our system.

We conclude that Akt signaling activity is segregated into distinct Akt pools, one acting upon Gsk-3β that depends on Appl1, and another activating Tsc2 independently of Appl1.

Akt and GSK-3β, but Not TSC2, Colocalize with APPL1 on Endosomes

If APPL1 regulates Akt activity, one might expect at least a fraction of Akt to colocalize with APPL1 on the same endosomes. To test for this, we exploited the optimal spatial resolution and responsiveness to growth factors of cultured mammalian cells. HeLa cells were starved and stimulated with IGF-1, an established inducer of Akt activity, in a time course experiment. To facilitate the visualization of proteins associated with cytoplasmic membranes, we permeabilized cells prior to fixation to release cytosol (Stenmark et al., 1994), and detected endogenous Akt and APPL1 by immunolabeling. Precise quantification of total Akt intensity and APPL colocalization using automated image analysis software (Rink et al., 2005) revealed that already 1 min

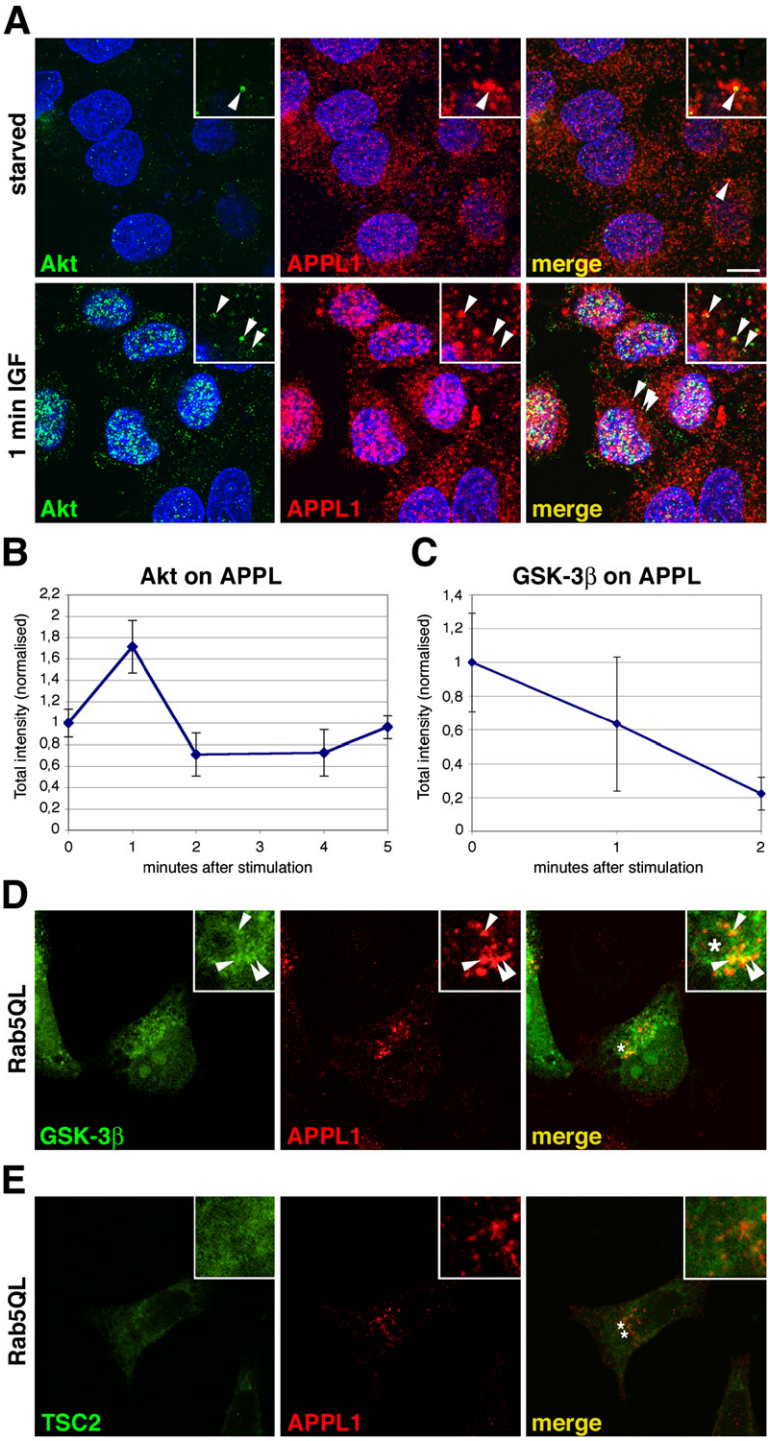

Figure 6. Akt and GSK-3β, but Not TSC2, Colocalize with APPL1 on Endosomes

(A) Colocalization of endogenous Akt and APPL1 in permeabilized HeLa cells before (upper panels) and after 1 min IGF-1 stimulation (lower panels).

(B) Quantitative evaluation of anti-Akt fluorescence intensity on APPL endosomes. HeLa cells were starved and stimulated with IGF-1 for 1, 2, 4, and 5 min. Akt fluorescence intensities were normalized against the fluorescence value in the starved condition. Error bars represent SEM.

(C) Quantitative evaluation of GFP-GSK-3β fluorescence intensity on APPL1 endosomes after extraction, carried out as described in (A).

(D) HeLa cells double-transfected with GFP-GSK-3β and a constitutively active form of Rab5, $Rab5^{Q79L}$, immunolabeled with anti-APPL1 (in red). $Rab5^{Q79L}$ induces the formation of enlarged endosomes (labeled by an asterisk, merge panel) and recruites APPL1 and GSK-3β.

(E) HeLa cells double transfected with flag-TSC2 and Rab5QL and immunolabeled with anti-flag (in green) and anti-APPL1 (in red). Asterisks label $Rab5^{Q79L}$ -induced enlarged endosomes (labeled by asterisks, merge panel). The TSC2 signal in insets has been enhanced to demonstrate that even the residual TSC2 remained after extraction shows a localization pattern different from Appl1.

(A, D, and E) Arrowheads point to endosomes that show colocalization, magnified in insets. The scale bar (in [A]) represents 10 μm.

after stimulation the total intensity of Akt signal on APPL endosomes increased by a factor 1.71 ± 0.24 ($p < 0.01$, Figures 6A and 6B). At this time point, 11.3% ± 1.4% of Akt-positive membranes also stained for APPL1. However, this increase was transient (Figure 6B). Similar results were obtained using a GFP-Akt construct (data not shown). Since the number of APPL endosomes did not significantly change with time, the observed changes are due to fast recruitment of Akt onto, and subsequent dissociation from, APPL endosomes.

The presence of Akt at APPL endosomes raises the question of whether its signaling specificity could relate to the localization of its downstream effectors to APPL endosomes. To test this idea, we explored the localization of flag-TSC2 or GFP-GSK-3β (due to poor detection of the endogenous proteins by antibody staining) after IGF-1 stimulation. The majority of TSC2 fluorescence was eliminated by extraction because of the prevailing solubility of the protein and the residual labeling did not show any colocalization with APPL1, both under starvation and stimulated conditions (data not shown). In contrast, GSK-3β was consistently seen to colocalize with APPL1 under starvation albeit at relatively low levels (4.1% ± 0.8% SEM). Localization of GSK-3β to canonical EEA1-positive early endosomes was, however, significantly lower (only 1.3% ± 0.9% SEM), indicating that GSK-3β is enriched on APPL endosomes relative to other endosomal compartments. Moreover, GSK-3β rapidly dissociated from APPL1 endosomes, its relative intensity dropping to 22,4% ± 9.7% within the first 2 min (Figure 6C).

Because of this highly dynamic behavior, we stabilized the localization of signaling components to the APPL compartment by expressing the constitutively active $Rab5^{Q79L}$ mutant that induces formation of enlarged endosomes (Stenmark et al., 1994) and accumulation of APPL1 (Miaczynska et al., 2004a). We

observed a striking recruitment of GSK-3β (Figure 6D), but not TSC2 (Figure 6E), to APPL-positive large endosomes. The differential presence of GSK-3β and TSC2, in agreement with their activity status in Appl morphants, strongly supports endosomal segregation as (part of) the mechanism underlying signaling specificity.

Membrane-Bound Ectopic Appl1 Induces Dysmorphic Phenotypes Resembling Akt Overexpression

To determine whether Appl1 is also rate-limiting during development, we performed gain-of-function (GOF) studies. Overexpression of Appl1 caused developmental delay and with high penetrance a number of dysmorphic phenotypes, such as severely swollen yolk extension, slightly swollen telencephalon, blown-up blood island and, later in development, sharp kinks in body axes (Figures 7A and 7B). As expected, these GOF phenotypes can be suppressed by coinjection of MO1B that targets the *appl1* mRNA (data not shown).

Because overexpression of Appl1 induces a mild increase of P-Akt levels (Figure 5A) and hyperphosphorylation of Akt2 in cultured cells (Yang et al., 2003), we asked whether not only Appl1 loss of function but also GOF phenotypes are mediated by this kinase. Injection of *akt2* mRNA (300–600 pg) indeed led to increased levels of both total and P-Akt (Figure 7C). Morphologically, Akt2 overexpressing embryos phenocopied Appl1-overexpression embryos, exhibiting swollen yolk extension and telencephalon, blown up blood island (Figure 7C) as well as later in development often bent or kinked body axes (data not shown), raising the possibility that the Appl1 GOF phenotypes result from an ectopically activated Appl-Akt pathway.

We next addressed whether Appl1 evokes its GOF phenotypes from its endosomal compartment, in the cytosol or within the nucleus. We therefore engineered three Appl1 variants with different subcellular destinations. First, to force Appl1 into the nucleus, we fused a nuclear localization signal (NLS-Appl1) to its N-terminus. Second, to prevent Appl1 from entering the nucleus, we fused a bulky fluorescent tag (Appl1-Venus) to the protein, since this hinders nuclear translocation of human APPL1 (Miaczynska et al., 2004a). Third, we designed a cytosolic Appl1 variant unable of localizing to the endosome membrane, by introducing three point mutations in the BAR domain (R147A, K153A and K155A) that together disrupt its membrane-association (Figure S5A). Figures 7D–7F show that these Appl1 variants, with 100% penetrance, exhibited the expected properties. Neither nuclear nor soluble Appl1 proteins affected normal development nor did they induce the aforementioned GOF phenotypes when expressed under the same conditions as WT Appl1 (Figures 7D and 7F). In contrast, the endosomal Appl1 variant (Appl1-Venus) caused developmental delay and GOF phenotypes indistinguishable from those evoked by the WT protein (Figure 7E).

These results suggest that hyperactivation of the Appl1-Akt signaling pathway induces the dysmorphic phenotypes described, and demonstrate that such signaling function depends on its endosomal localization.

Endosomal Localization Is Essential for Appl1-Mediated Akt Activation and Cell Survival Signaling

Finally, we wished to obtain evidence that Appl1 evokes its survival signaling activity from its endosomal membrane, 1) by assessing the dependence of Akt activity on the endosomal localization of Appl1 and Appl2) by determining whether the apoptotic phenotype induced by Appl1 knockdown can be rescued by either the nuclear NLS-Appl1 (see Figure 7D) or the soluble triM-Appl1 mutant (see Figure 7F). Western blot analysis of extracted WT, morphant and mRNA-coinjected morphant fish demonstrated that Akt activity (P-Akt) can only be restored by endosomal, but neither by nuclear nor soluble Appl1 proteins (Figure 7G). In perfect agreement, analyses of fish injected with morpholino and either NLS- or triM-Appl1 encoding mRNAs, carried out under the same conditions as the successful rescue experiment using *appl1* WT mRNA, showed that these mutants failed to rescue apoptosis (Figure 7H). Taken together, our data suggest that endosomal localization of Appl1 is both necessary and sufficient for Akt survival signaling.

DISCUSSION

The ability of cells to translate extracellular signals into appropriate cellular responses depends on a complex signaling network. Whereas many signaling modules have been identified, our knowledge about their regulatory mechanisms is still limited. Signaling from endocytic platforms is thought to contribute to signaling specificity and insulation (Miaczynska et al., 2004b; Teis and Huber, 2003), but concrete evidence in vivo has been lacking so far. Here, we report that Appl1 regulates the activity of Akt and, importantly, its downstream signaling specificity from an endosomal compartment, with profound implications for development of multicellular organisms.

The APPL Signaling Platform

Based on our data, the current model of Akt regulation and specificity needs to be refined to take into account the contribution of APPL endosomes to the signaling mechanisms. Previously, Akt phosphorylation at Thr308 has been reported to uncouple signaling to FoxO1/3 transcription factors from other Akt effectors (reviewed in Polak and Hall, 2006). With APPL1, we now provide a crucial missing link between the dependence on Rab5 for the activity of Akt (Hunker et al., 2006; Su et al., 2006) and its functional specification. We propose that receptors internalized into APPL endosomes are exposed to a molecular membrane environment enriched in selected signaling factors, thus "channeling" signaling flow downstream of Akt to evoke cell survival uncoupled from growth and proliferation. We envisage a platform for selective recruitment and activation of signaling components. The protein and lipid composition of such platform needs to be thoroughly established but it is likely to depend on a combinatorial use of protein-protein and protein-lipid interactions. For example, APPL proteins utilize coincidence detection of membrane curvature (Peter et al., 2004) and presence of Rab5 to achieve proper organelle targeting. Rab5 alone is not sufficient (Miaczynska et al., 2004a) since APPL localization also crucially depends on its BAR domain (Figures 7 and S5B). A similar combinatorial use of binding sites is likely to ensure the recruitment of downstream signaling components such as Akt and GSK-3β. However, such recruitment appears to be transient, suggesting that dynamic interactions rather than stable signaling complexes on the Appl endosomes account for signal propagation via Akt and GSK-3β. The precise kinetics and biochemical

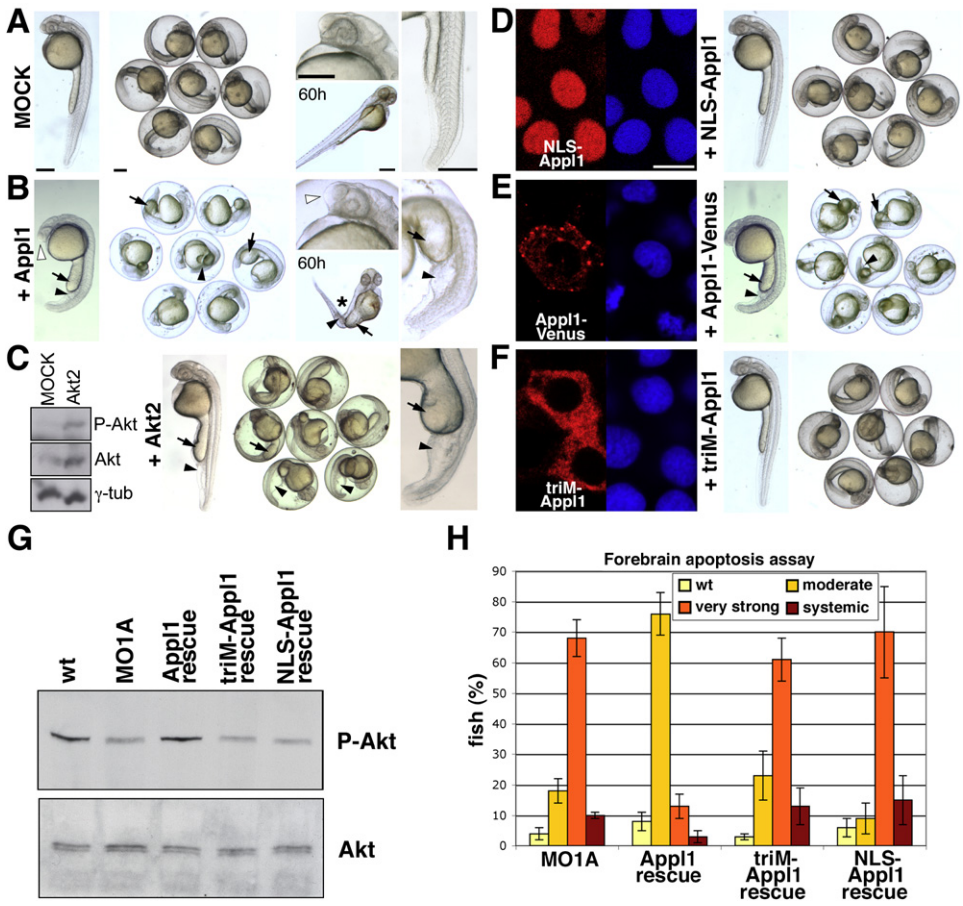

Figure 7. Appl1-Mediated Gain-of-Function Phenotypes and Survival Signaling Depend on Its Endosomal Localization

(A) Morphology of control embryos, injected with buffer only.

(B) Morphology of embryos injected with 500 pg *appl1* mRNA. Note the thick yolk extension (arrows), blown up blood island (black arrowheads), and swollen telencephalon (white arrowheads).

(C) Western blot analysis and morphology of embryos overexpressing Akt2. Total and P-Akt levels are elevated in embryos that have been injected with 300 pg *akt2* mRNA. γ-tubulin (γ-tub) was used as a loading control. The embryo on the left has received 300 pg mRNA, others 600 pg. Note thick yolk extensions (arrows) and blown up blood island (black arrowheads).

(D) Appl1 fused to a Nuclear Localization Signal (NLS-Appl1) is targeted to the nucleus and does not evoke phenotypes.

(E) Appl1 fused to a bulky tag (Appl1-Venus) is excluded from the nucleus and causes phenotypes identical to the one evoked by overexpression of WT Appl1 (B).

(F) Appl1 carrying the R147A, K153A, K155A triple mutation (triM-Appl1) fails to associate with endosomes and does not evoke phenotypes.

(A–F) Embryos after 26 hr of development, unless labeled otherwise. The scale bars (in [A]) represent 250 μm.

(D–F) Localization of three engineered Appl1 proteins and morphology of embryos injected with 500 pg of mRNA, respectively. Anti-Appl1 (red) and nuclear draq5 staining (blue). The scale bar (in [D]) represents 10 μm.

(G) Evaluation of Akt activity in WT, Appl1 knockdown (MO1A: 8 ng MO1A), and embryos in which a rescue with the WT *appl1* mRNA (Appl1 rescue: 8 ng MO1A + 75 pg *appl1* mRNA lacking MO1A binding site), the soluble triM-Appl1 (triM-Appl1 rescue: 8 ng MO1A + 75 pg *triM-appl1* mRNA lacking MO1A binding site), or the nuclear NLS-Appl1 mutant (NLS-Appl1 rescue: 8 ng MO1A + 75 pg *NLS-appl1* mRNA lacking MO1A binding site) has been attempted.

(H) Forebrain apoptosis assay: Quantitative comparison of apoptosis in embryos manipulated as in (G). Embryos were assigned to one of the four indicated cell death categories. Evaluation of Appl1 knockdown and WT *appl1* mRNA–rescued embryos (Figure 4G), are shown again for comparison. Total amount of embryos scored: $n^{\text{triM-Appl1 rescue}} = 567$, $n^{\text{NLS-Appl1 rescue}} = 502$. Error bars indicate SD amongst five experiments.

(G and H) Experiments have been carried out on 54 hr zebrafish embryos.

features of these interactions need to be evaluated using ad hoc developed quantitative live cell imaging techniques (e.g., FLIM).

General Relevance of Endosomal APPL for Receptor Trafficking and Signaling

Which signaling receptors may exploit APPL1 and its endosomal compartment to evoke specialized signaling responses among the cellular repertoire of downstream pathways? APPL1 has been reported to interact directly with transmembrane receptors of different classes. Moreover, APPLs bind to the PDZ domain protein GIPC (Lin et al., 2006; Varsano et al., 2006 and our unpublished data), an adaptor that potentially links them to an even larger set of receptors (Katoh, 2002). This suggests that trafficking through APPL endosomes is a widely utilized route by which

receptors may evoke signaling specificity. Several APPL-associated receptors have already been reported to signal through Akt (e.g., Caruso-Neves et al., 2006; Lin et al., 2006; Mao et al., 2006; Nechamen et al., 2004; Varsano et al., 2006), suggesting that APPL endosomes may transmit survival signals upon a variety of stimuli.

APPL1 has been recently shown to coimmunoprecipitate with the FSH receptor, and so did Akt2 and FoxO1a (Nechamen et al., 2007), another direct Akt target involved in survival signaling (Manning and Cantley, 2007). These observations and the phenotypes reported here raise the possibility that Akt-mediated survival pathways other than GSK-3β also depend on APPL endosomes. Furthermore, APPL1 and APPL2 are also present in the nucleus and have been shown to interact with the nucleosome remodelling and histone deacetylase complex NuRD/MeCP1 (Miaczynska et al., 2004a). This argues for additional roles of APPL proteins in signaling pathways leading to chromatin remodeling that remain to be explored. What may be the degree of functional redundancy between the two homologous Appl1 and Appl2 proteins? Double-knockdown experiments indicated that low-dose *appl1*+*appl2* morphants exhibit enhanced apoptosis in the olfactory system and in the neural tube (see Figure S6), but the majority of tissues does not change in comparison to single knockdown conditions. Appl1 and Appl2 may thus exhibit both redundant and nonredundant functions, in a tissue-dependent manner. Whereas a certain degree of redundancy seems indeed likely according to the high similarity between the two proteins, a recent study (Erdmann et al., 2007) has established the Lowe Syndrome protein OCRL as a direct partner of APPL1, not APPL2. Interestingly, Lowe syndrome is characterized by kidney defects, correlating with expression and survival function of Appl1, not Appl2, in the zebrafish pronephros.

Beyond embryonic development, APPL-dependent segregation of Akt downstream pathways would also be expected to contribute to tissue maintenance and homeostasis. At such stages, there is a requirement to translate trophic factors into cell survival signals, without evoking growth and proliferation. In light of the oncogenic potential of Akt signaling (Cheng et al., 2005), uncoupling of Akt-mediated survival might therefore be indispensable to avoid tumor growth. APPL1 and its endosomal compartment may play an important role in this scenario, as supported by (1) deregulation of the APPL1 interacting partner GIPC in some tumors (Katoh, 2002; Muders et al., 2006), and (2) our observation that increased Appl1 levels in zebrafish phenocopy effects of enhanced Akt2 signaling (see Figure 7), known to be sufficient for cell transformation (Cheng et al., 2005). The regulatory function of APPL1 on Akt signaling and its mediated substrate specificity discovered here may therefore render human APPL1 a particularly interesting target for cancer therapy.

EXPERIMENTAL PROCEDURES

Molecular Cloning and Antibodies

Constructs, primer sequences, antibodies, and staining reagents are described in Supplemental Experimental Procedures.

Fish Maintenance and Manipulation

Zebrafish were maintained at 28.5°C under standard conditions. Wt strains Gol and AB were used. mRNAs were transcribed in vitro using the SP6 Message Machine kit (Ambion, Austin, TX). MOs were designed and synthesized by Gene-Tools LLC, Philomath, OR. Their sequences, details on the injection procedure and information about TILLING are provided as Supplemental Experimental Procedures. For treatment with PI3K inhibitors (Calbiochem, San Diego), 53 hr fish embryos were transferred for 1 hr into fish water containing 50 μM LY294002 or 500 nM Wortmannin, which had no effect on fish behavior.

GST Pull-Down Assay

Preparation of GST-Rab5C and the assay were performed as previously described (Christoforidis et al., 1999).

Extracts and Western Blot Analysis

ZF4 cells were scraped in SDS loading buffer. Embryos of up to 24 hr of development were deyolked by passing them four times through a 200 μl eppendorf pipette tip and subsequent incubation in 0.5× Ringer solution [55 mM NaCl, 1.8 mM KCl, 1.25 mM $NaH(CO)^3$, Protease Inhibitor Cocktail], for 5 min under rotation. Embryos were spun down and hot 1× SDS loading buffer was added. To extract embryos >24 hr, hot 1× SDS loading buffer was added and the suspension was grinded vigorously using an Eppendorf pistil. SDS-PAGE and WB analysis (using TBS buffer) have been performed according to standard procedures.

Fish In Situ Hybridizations and Immunohistochemisty

The *appl1* and *appl2* Digoxigenin-labeled antisense and sense probes were in vitro transcribed using a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany). Whole-mount in situ hybridizations (ISH) and immunochemistry (IHC) were performed according to standard protocols and revealed with either fluorescently labeled or Alkaline Phosphatase-conjugated secondary antibodies, the latter one followed by NBT/BCIP staining.

Cell Culture, Immunocytochemistry, and Data Evaluation

Zebrafish ZF4 cells (ATCC #CRL-2050) were cultured according to ATCC's instructions. HeLa cells were grown and transfected according to standard procedures. Cells were starved in serum-free medium overnight, treated with 100 ng/ml IGF-1 (Novozymes GroPep, Adelaide, Australia) for different time intervals at 37°C, and permeabilized in 0.01% saponin, 80 mM PIPES (pH 6.8), 5 mM EGTA, and 1 mM $MgCl_2$ for 1 min to leak out the cytosol (Stenmark et al., 1994), prior to fixation in 4% paraformaldehyde/PBS. Cells were processed further for immunofluorescence analysis according to standard procedures and imaged using the same confocal settings. Data from three independent experiments were subjected to data analysis by the computer-assisted, automated image analysis program Motion-Tracking (Rink et al., 2005). For the Akt time course, 70 images/condition have been evaluated, representing more than 600 cells, 400.000 APPL, and 180.000 Akt endosomes. For GSK-3β, 100 images in total representing about 400 cells, 90.000 APPL, 83.000 EEA1, and 65.000 GSK-3β vesicles have been evaluated. P values were determined by Student's t test.

Apoptotic Staining and Forebrain Apoptosis Assay

Dechorionated embryos were incubated for 30 min in fish water containing 5 μg/ml apoptotic dye AO (Sigma Chemical Co., St. Louis, MO), followed by eight washes. AO-stained embryos were imaged and/or evaluated live. Embryos were assigned to one of four apoptotic categories. More details can be found in Supplemental Experimental Procedures.

ACCESSION NUMBERS

Zebrafish Appl1 and Appl2 sequences have been deposited in GenBank under the codes EU053152 and EU053153, respectively.

SUPPLEMENTAL DATA

Supplemental Data include six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/133/3/486/DC1/.

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REFERENCES

Abrams, J.M., White, K., Fessler, L.I., and Steller, H. (1993). Programmed cell death during Drosophila embryogenesis. Development *117*, 29–43.

Bardwell, L. (2006). Mechanisms of MAPK signalling specificity. Biochem. Soc. Trans. *34*, 837–841.

Brazil, D.P., Yang, Z.Z., and Hemmings, B.A. (2004). Advances in protein kinase B signalling: AKTion on multiple fronts. Trends Biochem. Sci. *29*, 233–242.

Caruso-Neves, C., Pinheiro, A.A., Cai, H., Souza-Menezes, J., and Guggino, W.B. (2006). PKB and megalin determine the survival or death of renal proximal tubule cells. Proc. Natl. Acad. Sci. USA *103*, 18810–18815.

Cheng, J.Q., Lindsley, C.W., Cheng, G.Z., Yang, H., and Nicosia, S.V. (2005). The Akt/PKB pathway: molecular target for cancer drug discovery. Oncogene *24*, 7482–7492.

Christoforidis, S., Miaczynska, M., Ashman, K., Wilm, M., Zhao, L., Yip, S.C., Waterfield, M.D., Backer, J.M., and Zerial, M. (1999). Phosphatidylinositol-3-OH kinases are Rab5 effectors. Nat. Cell Biol. *1*, 249–252.

Cole, L.K., and Ross, L.S. (2001). Apoptosis in the developing zebrafish embryo. Dev. Biol. *240*, 123–142.

Cooper, M.S., Szeto, D.P., Sommers-Herivel, G., Topczewski, J., Solnica-Krezel, L., Kang, H.C., Johnson, I., and Kimelman, D. (2005). Visualizing morphogenesis in transgenic zebrafish embryos using BODIPY TR methyl ester dye as a vital counterstain for GFP. Dev. Dyn. *232*, 359–368.

Dhillon, A.S., Hagan, S., Rath, O., and Kolch, W. (2007). MAP kinase signalling pathways in cancer. Oncogene *26*, 3279–3290.

Di Guglielmo, G.M., Le Roy, C., Goodfellow, A.F., and Wrana, J.L. (2003). Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. Nat. Cell Biol. *5*, 410–421.

Dumont, J.E., Pecasse, F., and Maenhaut, C. (2001). Crosstalk and specificity in signalling. Are we crosstalking ourselves into general confusion? Cell. Signal. *13*, 457–463.

Erdmann, K.S., Mao, Y., McCrea, H.J., Zoncu, R., Lee, S., Paradise, S., Modregger, J., Biemesderfer, D., Toomre, D., and De Camilli, P. (2007). A role of the Lowe syndrome protein OCRL in early steps of the endocytic pathway. Dev. Cell *13*, 377–390.

Hoeller, D., Volarevic, S., and Dikic, I. (2005). Compartmentalization of growth factor receptor signalling. Curr. Opin. Cell Biol. *17*, 107–111.

Hoepfner, S., Severin, F., Cabezas, A., Habermann, B., Runge, A., Gillooly, D., Stenmark, H., and Zerial, M. (2005). Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B. Cell *121*, 437–450.

Hunker, C.M., Kruk, I., Hall, J., Giambini, H., Veisaga, M.L., and Barbieri, M.A. (2006). Role of Rab5 in insulin receptor-mediated endocytosis and signaling. Arch. Biochem. Biophys. *449*, 130–142.

Husi, H., Ward, M.A., Choudhary, J.S., Blackstock, W.P., and Grant, S.G. (2000). Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. Nat. Neurosci. *3*, 661–669.

Jope, R.S., and Johnson, G.V. (2004). The glamour and gloom of glycogen synthase kinase-3. Trends Biochem. Sci. *29*, 95–102.

Katoh, M. (2002). GIPC gene family. Int. J. Mol. Med. *9*, 585–589.

Kholodenko, B.N. (2003). Four-dimensional organization of protein kinase signaling cascades: the roles of diffusion, endocytosis and molecular motors. J. Exp. Biol. *206*, 2073–2082.

Le Roy, C., and Wrana, J.L. (2005). Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. Nat. Rev. Mol. Cell Biol. *6*, 112–126.

Lin, D.C., Quevedo, C., Brewer, N.E., Bell, A., Testa, J.R., Grimes, M.L., Miller, F.D., and Kaplan, D.R. (2006). APPL1 associates with TrkA and GIPC1 and is required for nerve growth factor-mediated signal transduction. Mol. Cell. Biol. *26*, 8928–8941.

Liu, J., Yao, F., Wu, R., Morgan, M., Thorburn, A., Finley, R.L., Jr., and Chen, Y.Q. (2002). Mediation of the DCC apoptotic signal by DIP13 alpha. J. Biol. Chem. *277*, 26281–26285.

Manning, B.D., and Cantley, L.C. (2007). AKT/PKB signaling navigating downstream. Cell *129*, 1261–1274.

Mao, X., Kikani, C.K., Riojas, R.A., Langlais, P., Wang, L., Ramos, F.J., Fang, Q., Christ-Roberts, C.Y., Hong, J.Y., Kim, R.Y., et al. (2006). APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. Nat. Cell Biol. *8*, 516–523.

McCallum, C.M., Comai, L., Greene, E.A., and Henikoff, S. (2000). Targeted screening for induced mutations. Nat. Biotechnol. *18*, 455–457.

Miaczynska, M., Christoforidis, S., Giner, A., Shevchenko, A., Uttenweiler-Joseph, S., Habermann, B., Wilm, M., Parton, R.G., and Zerial, M. (2004a). APPL Proteins Link Rab5 to Nuclear Signal Transduction via an Endosomal Compartment. Cell *116*, 445–456.

Miaczynska, M., Pelkmans, L., and Zerial, M. (2004b). Not just a sink: endosomes in control of signal transduction. Curr. Opin. Cell Biol. *16*, 400–406.

Mitsuuchi, Y., Johnson, S.W., Sonoda, G., Tanno, S., Golemis, E.A., and Testa, J.R. (1999). Identification of a chromosome 3p14.3–21.1 gene, APPL, encoding an adaptor molecule that interacts with the oncoprotein-serine/threonine kinase AKT2. Oncogene *18*, 4891–4898.

Muders, M.H., Dutta, S.K., Wang, L., Lau, J.S., Bhattacharya, R., Smyrk, T.C., Chari, S.T., Datta, K., and Mukhopadhyay, D. (2006). Expression and regulatory role of GAIP-interacting protein GIPC in pancreatic adenocarcinoma. Cancer Res. *66*, 10264–10268.

Nechamen, C.A., Thomas, R.M., Cohen, B.D., Acevedo, G., Poulikakos, P.I., Testa, J.R., and Dias, J.A. (2004). Human follicle-stimulating hormone (FSH) receptor interacts with the adaptor protein APPL1 in HEK 293 cells: potential involvement of the PI3K pathway in FSH signaling. Biol. Reprod. *71*, 629–636.

Nechamen, C.A., Thomas, R.M., and Dias, J.A. (2007). APPL1, APPL2, Akt2 and FOXO1a interact with FSHR in a potential signaling complex. Mol. Cell. Endocrinol. *260–262*, 93–99.

Nowak, M., Koster, C., and Hammerschmidt, M. (2005). Perp is required for tissue-specific cell survival during zebrafish development. Cell Death Differ. *12*, 52–64.

Pal, A., Severin, F., Lommer, B., Shevchenko, A., and Zerial, M. (2006). Huntingtin-HAP40 complex is a novel Rab5 effector that regulates early endosome motility and is up-regulated in Huntington's disease. J. Cell Biol. *172*, 605–618.

Panopoulou, E., Gillooly, D.J., Wrana, J.L., Zerial, M., Stenmark, H., Murphy, C., and Fotsis, T. (2002). Early endosomal regulation of Smad-dependent signaling in endothelial cells. J. Biol. Chem. *277*, 18046–18052.

Pawson, T., and Scott, J.D. (2005). Protein phosphorylation in signaling–50 years and counting. Trends Biochem. Sci. *30*, 286–290.

Pelkmans, L., Burli, T., Zerial, M., and Helenius, A. (2004). Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. Cell *118*, 767–780.

Peter, B.J., Kent, H.M., Mills, I.G., Vallis, Y., Butler, P.J., Evans, P.R., and McMahon, H.T. (2004). BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. Science *303*, 495–499.

Polak, P., and Hall, M.N. (2006). mTORC2 Caught in a SINful Akt. Dev. Cell *11*, 433–434.

Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. Cell *122*, 735–749.

Robu, M.E., Larson, J.D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S.A., and Ekker, S.C. (2007). p53 activation by knockdown technologies. PLoS Genet *3*, e78. 10.1371/journal.pgen.0030078.

Shin, H.W., Hayashi, M., Christoforidis, S., Lacas-Gervais, S., Hoepfner, S., Wenk, M.R., Modregger, J., Uttenweiler-Joseph, S., Wilm, M., Nystuen, A., et al. (2005). An enzymatic cascade of Rab5 effectors regulates phosphoinositide turnover in the endocytic pathway. J. Cell Biol. *170*, 607–618.

Stenmark, H., Parton, R.G., Steele-Mortimer, O., Lutcke, A., Gruenberg, J., and Zerial, M. (1994). Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. EMBO J. *13*, 1287–1296.

Su, X., Lodhi, I.J., Saltiel, A.R., and Stahl, P.D. (2006). Insulin-stimulated Interaction between insulin receptor substrate 1 and p85alpha and activation of protein kinase B/Akt require Rab5. J. Biol. Chem. *281*, 27982–27990.

Teis, D., and Huber, L.A. (2003). The odd couple: signal transduction and endocytosis. Cell. Mol. Life Sci. *60*, 2020–2033.

Teis, D., Wunderlich, W., and Huber, L.A. (2002). Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev. Cell *3*, 803–814.

Varsano, T., Dong, M.Q., Niesman, I., Gacula, H., Lou, X., Ma, T., Testa, J.R., Yates, J.R., 3rd, and Farquhar, M.G. (2006). GIPC is recruited by APPL to peripheral TrkA endosomes and regulates TrkA trafficking and signaling. Mol. Cell. Biol. *26*, 8942–8952.

Weston, C.R., and Davis, R.J. (2001). Signal transduction: signaling specificity-a complex affair. Science *292*, 2439–2440.

Yang, L., Lin, H.K., Altuwaijri, S., Xie, S., Wang, L., and Chang, C. (2003). APPL suppresses androgen receptor transactivation via potentiating Akt activity. J. Biol. Chem. *278*, 16820–16827.