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The insulin signaling cascade from nematodes to mammals: insights into innate immunity of *Anopheles* mosquitoes to malaria parasite infection

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Abstract

As revealed over the past twenty years, the insulin signaling cascade plays a central role in regulating immune and oxidative stress responses that affect the life spans of mammals and two model invertebrates, the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. In mosquitoes, insulin signaling regulates key steps in egg maturation and immunity and likely affects aging, although the latter has yet to be examined in detail. Reproduction, immunity and aging critically influence the capacity of mosquitoes to effectively transmit malaria parasites. Current work has demonstrated that molecules from the invading parasite and the blood meal elicit functional responses in female mosquitoes that are regulated through the insulin signaling pathway or by cross-talk with interacting pathways. Defining the details of these regulatory interactions presents significant challenges for future research, but will increase our understanding of mosquito/malaria parasite transmission and of the conservation of insulin signaling as a key regulatory nexus in animal biology.

Keywords

insulin; *Anopheles*; *Plasmodium*; mosquito; malaria; aging; oxidative stress; innate immunity; nitric oxide; transforming growth factor; β

In all well-studied metazoans, metabolism, growth, and longevity are coordinately regulated via a nexus that involves signaling cascades activated by insulin and insulin-like growth factors (IGF). Although these pathways differ in downstream physiological effects and some of their signaling proteins, the proteins themselves and their scaffolding interactions are largely conserved among model organisms (Fig. 1), particularly in the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the mouse *Mus musculus*. This review will examine our current understanding of how the insulin and IGF signaling cascades (ISC) regulate immunity in these diverse model organisms. In addition, we will explore how recent

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research on three biomedically important mosquitoes links the ISC to regulation of metabolism, aging, and immunity to pathogens responsible for diseases such as malaria.

1. Insulin signaling, innate immunity and aging in *Caenorhabditis elegans*

In *C. elegans*, insulin signaling presumably is initiated by the binding of insulin-like peptides (ILPs), of which there are at least 38 [1], to a receptor tyrosine kinase, daf-2, that is an apparent homolog to the vertebrate insulin/IGF receptor. Based largely on interpretations from studies with various genetic mutants, daf-2 activates AGE-1, a phosphoinositide-3 kinase (PI-3K), which in turn activates Akt/protein kinase B. Importantly, activated Akt can directly phosphorylate daf-16, a Forkhead transcription factor ortholog, which inactivates daf-16 by excluding it from the nucleus [2]. In the absence of insulin signaling, unphosphorylated daf-16 can enter the nucleus and control transcription, indicating that the actions of daf-2 and daf-16 oppose each other. This fact is highlighted by mutants with opposed physiologies: *daf-2* loss-of-function mutants are long-lived and resistant to oxidative stress and bacterial infection, whereas *daf-16* loss-of-function mutants are short-lived and susceptible to oxidative stress and infection [3,4].

Key targets of daf-16 regulation include genes for mitochondrial manganese superoxide dismutase (MnSOD) and glutathione S-transferase, which are closely linked to aging in *C. elegans* and other organisms, and genes for numerous C-type lectins, which play critical roles in cell adhesion and pathogen recognition [5]. Downregulation of these gene targets in *daf-16* mutants would be predicted to enhance oxidative stress, which has been linked directly to aging in *C. elegans* [6,7], and decrease pathogen recognition [8]. Conversely, rescue of downregulated MnSOD has been shown to reverse accelerated aging and mortality due to oxidative stress [6]. Specifically, Melov et al. [6] reported that dietary supplementation of *C. elegans* with the SOD mimic EUK-8, which accumulates in mitochondria in mammalian cells [9], significantly increased the lifespan of normal *C. elegans*. Keaney et al. [9], however, reported that EUK-8 extends lifespan in oxidatively stressed, but not in unstressed nematodes. Despite these differences, studies of selected gene mutations in *C. elegans* generally support the “oxidative stress hypothesis of aging,” that is, both normal and accelerated aging and mortality are the result of accumulated oxidative damage. As such, when *daf-16* expression is activated, oxidative stress is reduced, and lifespan is extended. Conversely, when *daf-16* expression is inhibited, oxidative stress is enhanced, and lifespan is shortened.

In *C. elegans*, the intestine and germ tissue play pivotal roles in signaling crosstalk that are critical for normal development and aging. Indeed, the intestine has been defined as a “signaling center” from which overexpressed *daf-16* can completely restore the longevity of *daf-16* germline-deficient nematodes and significantly increase the lifespan of these mutants [10]. Libina et al. [10] proposed that overexpressed *daf-16* acts to control lifespan by downregulating a daf-2 agonist or by upregulating a daf-2 antagonist. Interestingly, the observations of Murphy et al. [11] support this hypothesis: expression of the insulin-like peptide gene *ins-7*, which encodes a putative daf-2 agonist, was elevated in nematodes in which *daf-16* expression was knocked down and reduced in nematodes engineered to overexpress *daf-16*. It is possible that reduced *ins-7* expression results in reduced secretion of bioactive peptide, and as such, is perceived by surrounding cells and tissues as reduced insulin signaling, which results in the spread of daf-16 activation. Such signal spread via the ISC is predicted to underlie the mechanisms of aging and would also be expected to enhance survivorship following immune challenge through coordination of local and systemic anti-pathogen responses.

2. Insulin signaling and physiology in *Drosophila melanogaster*

The conservation of an ISC pathway in *D. melanogaster* and its regulation of diverse processes have been explored largely through the phenotypic effects of genetic mutations, and they are

similar to those for *C. elegans*. For example, *D. melanogaster* homozygous for a P-element insertion of *chico*, the insulin receptor substrate gene, exhibited a 48% increase in lifespan relative to wild type flies [12]. Mutant flies also exhibited enhanced levels of SOD activity [12]. Similarly, flies with mutant alleles of the insulin receptor [13] and engineered to overexpress the Forkhead transcription factor, *dFOXO*, in the pericerebral fat body [14] exhibited enhanced longevity relative to wild type flies. Overexpression of *dFOXO* in pericerebral fat body also induced nuclear localization of dFOXO in peripheral fat body, which would be expected to repress insulin-dependent signaling in this tissue [14]. The latter observations would suggest that signal spread in fruit flies may mimic that observed in *C. elegans*.

In other studies, targeted ablation of neurosecretory cells that secrete ILPs in adult flies resulted in a 30–40% reduction in expression of the ILP genes *dilp2*, *dilp3*, and *dilp5*, increased lifespan by up to 33% in mated female flies, increased resistance to oxidative stress, and resulted in significantly higher levels of trehalose (64% increase), glycogen (44% increase), and lipids (10% increase) when compared with controls [15]. Bayne et al. [16] reported that flies genetically engineered to overexpress MnSOD and catalase in the mitochondria exhibited enhanced resistance to dietary hydrogen peroxide (H₂O₂) and exposure to hyperoxic conditions. However, the authors also noted that lifespan of the flies was decreased rather than increased, and this was associated with decreased physical activity and an age-related decrease in mitochondrial respiration, suggesting that physiological levels of reactive oxygen species are essential for normal biological processes and normal lifespan [16]. Together, these observations indicate that an ISC is involved in the regulation of aging in *D. melanogaster* through a complex interplay of stress resistance, ILP secretion, and apportionment of metabolic reserves.

In *C. elegans*, the connection between insulin signaling and innate immunity has been established through immune challenge of ISC mutants [3,4], but cause-and-effect between insulin signaling and innate immunity in *D. melanogaster* has not yet been established. A connection between aging and immunity, however, seems clear. Specifically, Zerofsky et al. [17] reported that female fruit flies exhibit immune senescence with age. Older female flies expressed higher levels of the antimicrobial peptide gene *dipterizin* relative to younger female flies when exposed to septic bacterial infections [17]. The response in older flies was attributed to persistent *dipterizin* transcription upon septic exposure, a phenomenon not observed in younger flies. In contrast, when older flies were challenged with killed bacteria, *dipterizin* induction levels were lower than induction levels observed in similarly treated younger flies, suggesting that older females had a reduced capacity to induce *dipterizin* [17]. The authors also showed that fecundity was reduced in immune-activated females via the “immune deficiency” or imd pathway, suggesting that maximum reproduction occurs in young flies, perhaps as a benefit from increased regulation of the immune response [17].

3. The ISC, innate immunity and aging in mammals

Only a single insulin/IGF receptor has been identified to date in the *C. elegans* and *D. melanogaster* genomes, whereas mammals possess distinct receptor tyrosine kinases for insulin and IGF-I, plus a third receptor type of unknown function. Caloric restriction, which increases life-span in rodents [18] and non-human primates (reviewed in [19]), reduces circulating levels of insulin and IGF-I (reviewed in [20]). In a manner similar to the connections between the ISC and aging in other organisms, there is evidence for a role for IGF-I in the control of longevity in mammals. Specifically, mice heterozygous for an IGF receptor mutation exhibited a 33% increase in longevity and upregulated MnSOD activity [21]. In mice, as in fruit flies, overexpression of catalase targeted to the mitochondria lengthened lifespan and reduced

oxidative tissue damage, including H₂O₂-induced aconitase inactivation and the development of mitochondrial deletions relative to control animals [22].

In general, IGF-I and insulin have broad activities in the mammalian innate immune system (reviewed in [23]), and these effects are dose-dependent. Normal circulating levels of mammalian IGF-I range from 0.8 – 1.0 µg/ml (0.11 – 0.13 µM; [24]) and those of insulin are ~ 0.4 ng/ml (6.7 x 10⁻⁵ µM or 0.067 nM; [25]). At concentrations as low as 50 ng/ml, IGF-I can augment the bactericidal activity of murine macrophages by increasing phagocytosis [26] and can synergize with lipopolysaccharide at concentrations from 0.13 – 1.3 nM to induce tumor necrosis factor (TNF)-α synthesis by human monocytes and murine macrophages [27]. In contrast to enhancement of immune responses by low concentrations of IGF-I, supraphysiological concentrations of insulin are required to induce similar effects. For example, a 24 h treatment of human monocytes and murine macrophages with 0.13 – 130 nM insulin did not induce TNF-α synthesis; induction was not observed until 24 h after treatment with 1.3 – 13 µM insulin, which is 1,000- to 10,000-fold higher than effective concentrations of IGF-I [27].

In contrast to the effects of IGF-I and insulin on innate immune cells, concentrations of IGF-I of 0.13 µM or greater and concentrations of insulin of 8.6 µM or greater reduced proliferation and antibody synthesis of adaptive immune cells (lymphocytes) relative to control cells *in vitro* [28]. The anti-inflammatory effects of insulin have been revisited recently in light of the beneficial effects of intensive insulin therapy (IIT) of critically ill patients [28,29]. These patients typically suffer from stress hyperglycemia, so the beneficial effects of IIT have not been clearly attributed to the prevention of hyperglycemia, which can induce oxidative stress, or to the purported anti-inflammatory effects of elevated levels of insulin *in vivo* [29,30].

Taken together, IGF-I and insulin can exert anti- and pro-inflammatory effects on immune cells, but these effects are mediated by low to physiological levels of IGF-I, while an excess of insulin is required to observe similar effects. At high concentrations, insulin can bind to the IGF-I receptor [31], suggesting that the similar effects of insulin and IGF-I at different doses are due to binding to the IGF-I receptor. This differential responsiveness is reflective of an increased biological complexity that may have evolved in mammals to focus the effects of circulating insulin on metabolism, whereas the effects of IGF-I were broadened to include growth regulation as well as regulation of longevity and immunity.

4. The ISC and nitric oxide in mammals: positive feedback and control of inflammation

Nitric oxide (NO), one of the smallest known biological molecules, regulates neuronal signaling, vascular physiology and numerous immune functions in mammals including direct pathogen killing (reviewed in [32]). Given the broad biological roles of NO, it is perhaps not surprising that control of insulin action has been connected functionally to NO synthesis in mammals. The synthesis of NO is catalyzed by a two-step oxidation of L-arginine by NO synthase (NOS) and, in mammals, three NOS isoforms have been identified. Two isoforms are commonly associated with endothelial and neuronal physiologies (eNOS, nNOS), while a third (iNOS) is highly inducible and often associated with immune function. These divisions, however, are somewhat artificial in that eNOS and nNOS are inducible by pathogen-associated molecules [33,34] and iNOS expression has been associated with signaling processes more typically associated with eNOS or nNOS [35].

Shankar *et al.* [36] showed that catheter delivery to rats of NG-monomethyl-L-arginine, a general inhibitor of NOS activity, induced hyperglycemia by inhibiting insulin secretion and action. These observations suggested that NO-dependent pathways potentiate insulin secretion

and action. Conversely, numerous studies have demonstrated insulin induction of NO synthesis, suggesting a positive feedback loop of NO-insulin-NOS induction. For example, Begum and Ragolia [37] reported that 100 nM insulin upregulated iNOS protein levels by nearly three-fold in rat vascular smooth muscle cells. Kim et al. [38] reported similar findings for rat osteoblast-like cells. Specifically, 10 nM insulin induced NO synthesis 1.3-fold and 100 nM insulin induced NO synthesis two-fold relative to controls [38]. Insulin induction of NO synthesis was inhibited by the MEK inhibitor PD98059 and induced by overexpression of Erk, indicating that insulin induced NO synthesis via activation of a MEK/Erk-dependent pathway [38]. In contrast to insulin, 100 nM IGF-1 increased NO synthesis by only 57% relative to controls, indicating that iNOS activity in osteoblast-like cells is significantly more sensitive to regulation by insulin than by IGF-1 [38]. In other studies, treatment of human endothelial cells with a concentration of insulin closer to physiological levels (1nM) induced NO synthesis via insulin receptor activation and activation of a downstream pertussis-sensitive GTP-binding protein and Erk 1/2-dependent signaling [39]. Taken together, these results indicate that insulin induces NO synthesis through multiple pathways and that NO synthesis is critical for insulin secretion and action. An insulin-NO positive feedback loop would be predicted to enhance the pro-inflammatory effects of insulin and to facilitate signal spread given the diffusion of NO across closely opposed cell membranes.

5. Endocrinology of insulin-like peptides and links to innate immunity in mosquitoes

The ISC in aedine and anopheline mosquitoes is highly conserved. Studies by Riehle and Brown [40-43] of *Aedes aegypti*, the yellow fever mosquito, revealed that a dose range of 1.7 – 85 μ M bovine insulin stimulated ecdysteroid production by ovaries isolated from female mosquitoes. This effect was transduced through the ISC, as determined with the use of inhibitors or activators of the insulin receptor, PI-3K and Akt [40,41]. Interestingly, the MEK inhibitor PD98059 had no effect on steroidogenesis, suggesting that the PI-3K/Akt branch of the ISC is activated for this physiological effect. Eight ILP genes have been identified in *Ae. aegypti*; one gene is distinctly IGF-like and expression patterns of the eight genes suggest division into growth factor and neurohormonal functions [44].

Genomic analyses of a second mosquito, *Anopheles gambiae*, the principal vector of malaria parasites (*Plasmodium* spp.) affecting humans in Africa, revealed genes encoding ILPs and proteins from both insulin signaling branches [45]. In subsequent studies, Krieger et al. [46] confirmed the existence of seven *An. gambiae* ILP genes, and as observed in *Ae. aegypti*, their developmental and tissue expression patterns have suggested specific growth factor and neuropeptide functions.

Studies with *Anopheles stephensi*, a major vector of human *Plasmodium* spp. in India and parts of the Middle East, have revealed that human insulin can induce NO synthesis in cultured cells and in the mosquito midgut [47]. Inducible NO synthesis in *An. stephensi* limits malaria parasite development [48] through the formation of inflammatory levels of toxic reactive nitrogen oxides [49,50] that likely induce parasite apoptosis in the mosquito midgut lumen [51]. As such, efforts to identify parasite-derived and other inducers of *AsNOS* expression are significant for the development of *AsNOS* or associated regulatory factor genes as targets for manipulation to enhance anti-parasite resistance. In *An. stephensi* cells *in vitro*, human insulin induced *AsNOS* expression two-fold relative to controls at 48 h after treatment, while provision of human insulin by artificial bloodmeal induced *AsNOS* expression in the midgut approximately two-fold at 6 h and four-fold at 3 h post-feeding relative to controls [47]. These observations suggested that insulin control of NO synthesis and immunity is conserved between *Anopheles* mosquitoes and mammals.

6. Insulin signaling and its impact on malaria transmission

The relevance of insulin signaling to malaria and, ultimately to the transmission of parasites by mosquitoes, is highlighted by studies of parasite infection in mammals. Infections with *Plasmodium* spp. in mice [52] and humans [53-56] induce hypoglycemia, which is often predictive of severe pathology and a fatal outcome. In mice, hypoglycemia has been causally linked to hyperinsulinemia [57]. In humans, hyperinsulinemia in response to parasite infection has been reported in Thai patients [53] and also during quinine therapy [58]. Average insulin levels in hyperinsulinemic malaria patients are approximately 1.6×10^{-4} μ M, with the highest recorded concentration at 4.7×10^{-4} μ M [53]. These levels contrast with blood levels of insulin in healthy patients that range from a fasting concentration of 1.7×10^{-5} μ M (0.1 ng/ml) to a non-fasting concentration of 5.9×10^{-4} μ M (3.4 ng/ml; [59]), indicating that blood levels of insulin can vary as much as 10- to 35-fold depending on nutrition and disease status.

The dysregulation of insulin levels and glycemic status during malaria parasite infection is complex, but has been attributed, in part, to the elaboration during infection of parasite-derived glycosylphosphatidylinositol or GPI, an abundant surface protein anchor in all *Plasmodium* species. Although GPIs are ubiquitous on eukaryotic cells, *Plasmodium* GPI differs structurally from GPI found on mammalian cells [60], is produced in vast excess of its use for protein anchoring [61] and also induces pro-inflammatory responses in mammalian host cells *in vitro* and *in vivo* [62,63]. Other studies revealed that *Plasmodium* GPI could induce lipogenesis and glucose oxidation in rat adipocytes and that injection of parasite GPI into mice could induce hypoglycemia [64]. These observations led to the hypothesis that *Plasmodium* GPIs were also insulin-mimetic. Subsequently, it was demonstrated that *Plasmodium* GPI exhibited signaling characteristics of the insulin extracellular mediator phosphoinositolglycan [65], which is released from host cell GPI by insulin stimulation of phosphatidylinositol-dependent phospholipase activity.

When a female mosquito ingests blood from a *Plasmodium*-infected host, a mix of erythrocytes, parasites, parasite-derived factors including GPI and an abundance of host-derived proteins including insulin is delivered to the midgut, a critical site for *Plasmodium* development in the mosquito (reviewed in [66]). After completing several stages of midgut development, malaria parasites invade the salivary glands as sporozoites, which are transmitted to the mammalian host in mosquito saliva. Parasite development requires 10 to 12 days and the average natural lifespan of *An. gambiae* is approximately two weeks. As such, this protracted development time presents a problem for the parasites since development and transmission must be completed before the mosquito dies or before the parasite is recognized and killed. Thus, mosquito aging and innate immunity are predicted to have significant impacts on parasite transmission.

Beier et al. [67] completed the first studies of the effects of human insulin on *P. falciparum* infection in *An. stephensi* and *An. gambiae*. In these studies, a high concentration of insulin (0.3 μ M) was provided to adult female mosquitoes in 10% sucrose from day 0 (newly emerged adults) to day 10 after feeding on an infectious blood meal [67]. In both mosquito species, parasite infection rates were 1.5- to 1.8-fold higher and oocyst densities were significantly higher in insulin-fed relative to control groups [67], mirroring the immunosuppressive effects of insulin in mammals at high concentrations.

In more recent studies, the effects of *P. falciparum* GPI (PfGPI) on anti-parasite NO synthesis and signal transduction in *An. stephensi* have revealed that, as in mammals, parasite GPI is an important early signal for parasite infection in mosquitoes [47]. Specifically, a blood meal containing physiological levels of PfGPI, relative to a non-supplemented blood meal, induced higher levels of phosphorylation of Akt and Erk and, within 1h after feeding, induced

AsNOS expression >2.5-fold in the midgut epithelium [47]. This suggests that early activation of ISC proteins contributes to the regulation of anti-parasite immunity in *An. stephensi* [47]. These findings, together with previous work, also indicate that insulin signaling in mosquitoes coordinately regulates immunity and at least one essential metabolic process, namely steroidogenesis, connecting evolutionary findings from model organisms to a system of significant human health importance.

In summary, current literature suggests that the ISC regulates physiological responses to oxidative stress and aging in *C. elegans*, *D. melanogaster* and mammals. Intriguing genetic correlations in nematodes suggest that insulin signaling regulation of immunity evolved in invertebrates. In mosquitoes, available data indicate that insulin signaling regulates reproduction and immunity and suggest that NO synthesis likely has multiple roles in the mosquito. At localized sites of low NO concentration, NO could facilitate signal spread to enhance the effects of ILPs on reproduction and immunity, while induction of high levels of NO and the formation of toxic nitrogen oxides [50] is consistent with an oxidative stress response that is directed toward the control of parasite infection. Limited data suggest that mosquito age does not have a significant effect on success of *P. falciparum* development, but a higher proportion of *An. gambiae* in a middle age range (4-7d) were infected relative to young (1-3d) and old (8-11d) age ranges [68]. Additional studies will be necessary to determine whether immune senescence occurs in mosquitoes as it does in fruit flies and to what extent aging in mosquitoes is regulated by insulin signaling. These studies are not only critical for our understanding of malaria parasite transmission by the insect vector, which is defined by the combined effects of age, physiological state, and immunity, but will also fill an important knowledge gap between nematodes and mammals in our understanding of the evolutionary conservation of insulin signaling.

The phenomenon of conserved of ISCs in the vertebrate and invertebrate hosts of pathogens leads to additional unanswered questions of mosquito-parasite biology. Specifically, it will be critical to determine how signals from malaria parasites, the blood meal, and the mosquito are integrated to produce a functional response. These factors, which may include parasite GPI, mammalian insulin or IGF, and mosquito NO and ILPs, may function synergistically along a dose-response continuum or they may act antagonistically so that the resource-intensive physiologies of reproduction and immunity are coordinately fine-tuned to maximize the benefits of blood feeding. Further, in mammals and nematodes, a second evolutionarily conserved growth factor signaling pathway regulated by the transforming growth factor (TGF)- β s, a large superfamily of proteins that includes growth factors and immunomodulatory cytokines, extensively intersects the insulin signaling pathway to regulate aging, oxidative stress, and immunity [69-73]. Like the insulin signaling pathway, the TGF- β signaling pathway regulates the NO-dependent immune response in *An. stephensi* [49,74].

Dissecting the processes of immunity, reproduction and aging in the mosquito, therefore, will require analysis of integrated signaling pathways that coordinately regulate them. In this sense, the biology of the mosquito and, indeed, of other bloodfeeding vectors offers a unique opportunity in the animal kingdom to examine to the fullest extent the functional consequences of conserved signaling pathways that intersect across more than 600 million years of evolution.

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Abbreviations

Erk	extracellular signal-regulated protein kinase
GPI	glycosylphosphatidylinositol
GRB2	growth factor receptor-bound protein 2
H₂O₂	hydrogen peroxide
IGF	insulin-like growth factor

IIT	intensive insulin therapy
ILP	insulin-like peptide
IRS	insulin receptor substrate
ISC	insulin/insulin-like growth factor signaling cascade
MAP kinase	mitogen-activated protein kinase
MEK	mitogen-activated or extracellular signal-regulated protein kinase
MnSOD	manganese superoxide dismutase
NOS	nitric oxide synthase
PDK1	phosphatidylinositol-dependent kinase 1
PI-3K	phosphatidylinositol 3-kinase
PTEN	phosphatase and tensin homolog
SHC	src homology domain-containing protein
SOS	son of sevenless protein
TGF-β	transforming growth factor- β
TNF-α	tumor necrosis factor- α

Figure 1. The insulin/insulin-like growth factor signaling cascade (ISC)

The ISC is initiated by an insulin-like peptide binding to the α subunit of the insulin receptor. This induces the autophosphorylation of the β subunit which in turn phosphorylates insulin receptor substrate 1 (IRS1). Phosphorylated tyrosine residues provide a binding site for the regulatory subunit of PI 3-kinase and SHC representing the major branching point between the PI 3-kinase (PI-3K)/Akt and Ras/Raf/MEK/Erk cascades. **PI 3-kinase/Akt cascade:** Upon PI 3-kinase binding to IRS-1 the catalytic subunit is released and travels to the cell membrane where it converts PI(4,5)P₂ into PI(3,4,5)P₃. PI(3,4,5)P₃ provides binding sites for both Akt and PDK1 and brings these proteins together. Phosphorylation of Akt by PDK1 activates the kinase activity of Akt, allowing it to phosphorylate extranuclear Forkhead transcription factors. These transcription factors can then translocate across the nuclear membrane and begin transcription of a variety of proteins. PTEN dephosphorylates PI(3,4,5)P₃ to PI(4,5)P₂ and thus acts to down-regulate the PI 3-kinase cascade. **MAP kinase cascade:** Upon activation the GRB2/SOS complex binds and activates Ras, which in turn recruits Raf to the cell membrane and activates it. The activated Raf subsequently activates MEK, which in turn activates Erk. Erk can then enter the nucleus to phosphorylate and activate transcription factors such as Elk-1.