

Non-canonical dynamic mechanisms of interaction between the p66Shc protein and Met receptor

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Met receptor tyrosine kinase (RTK) is known to bind to the three distinct protein isoforms encoded by the *ShcA* (*Shc*) gene. Structure–function studies have unveiled critical roles for p52Shc-dependent signalling pathways in Met-regulated biological functions. The molecular basis of the interaction between the Met and p52Shc proteins is well-defined, but not for the longest protein isoform, p66Shc. In the present study, co-immunoprecipitation assays were performed in human embryonic kidney 293 (HEK293) cells, transiently co-transfected with Met and p66Shc mutants, in order to define the molecular determinants involved in mediating Met–p66Shc interaction. Our results show that p66Shc interacts constitutively with the receptor Met, and the Grb2 (growth factor receptor-bound protein-2) and Gab1 (Grb2-associated binder-1) adaptor proteins. Although its phosphotyrosine-binding domain (PTB) and Src homology 2 (SH2) domains co-ordinate p66Shc binding to non-activated Met receptor, these phosphotyrosine-binding modules, and its collagen homology domain 2 (CH2) region, exert negative constraints.

In contrast, p66Shc interaction with the activated Met depends mainly on the integrity of its PTB domain, and to a lesser extent of its SH2 domain. Even though not required for the recruitment of p66Shc, tyrosine phosphorylation of p66Shc by activated Met enhances these interactions by mechanisms not reliant on the integrity of the Met multisubstrate-binding site. In turn, this increases phosphotyrosine-dependent p66Shc–Grb2–Gab1 complex formation away from the receptor, while blocking Grb2 and Gab1 recruitment to activated Met. In conclusion, we identify, for the first time, a novel non-canonical dynamic mode of interaction between Met and the p66 protein isoform of Shc and its effects on rewiring binding effector complexes according to the activation state of the receptor.

Key words: adaptor protein, Grb2-associated binder-1 (Gab1), growth factor receptor-bound protein-2 (Grb2), Met receptor, p66Shc, phosphotyrosine-binding domain.

INTRODUCTION

When activated, cell-surface growth factor receptor tyrosine kinases (RTKs) become phosphorylated on a number of tyrosine (Tyr) residues. Many of these phospho-Tyr residues located within the cytoplasmic tail of the receptor create binding sites for proteins containing Src homology 2 (SH2) and phospho-Tyr-binding (PTB) domains, which both recognize phospho-Tyr residues within the context of specific adjacent amino acids. Among proteins recruited to activate RTKs are the adaptor proteins, including growth factor receptor-bound protein-2 (Grb2) and Src homology collagen (Shc) proteins. These proteins are devoid of catalytic activity but contain multiple protein–protein interaction motifs and domains. As such, they mediate assembly of specific protein complexes to RTKs that relay fine-tuned downstream regulation of diverse signalling pathways and biological responses (reviewed in [1,2]).

In mammals, the family of Shc adaptor proteins includes four members (ShcA–ShcD) that share a similar domain organization. The *ShcA* gene encodes three distinct protein isoforms, p66, p52 and p46Shc (referred to in the present paper as Shc). The p52 and p46Shc isoforms derive from the same transcript via usage of two distinct translation initiation codons, and are ubiquitously expressed. By contrast, the expression of the p66Shc splicing

isoform is more restricted to epithelial cells [3]. All Shc proteins encompass a central proline-rich collagen homology domain (CH1) that is flanked by N-terminal PTB and C-terminal SH2 domains [2]. In addition, p66Shc contains an extra N-terminal proline-rich CH2 domain [4]. The Shc adaptor proteins are understood to interact with activated RTKs, mainly via their PTB domain. In turn, this triggers the phosphorylation of Tyr residues present within consensus binding sites for the Grb2 SH2 domain (pYXNX), which are located in the CH1 domain of Shc proteins (Tyr-239/240/317 or Tyr-349/350/423 in mouse p52Shc and p66Shc respectively), thereby allowing the activation of the mitogenic Ras/mitogen-activated protein kinase (MAPK) and survival phosphoinositide 3-kinase (PI3K)/Akt cascades [2]. This model for RTK-mediated recruitment of Shc is mainly based on structural studies of the p52 isoform of Shc, which is the first discovered and best-characterized Shc family member. However, the mechanisms by which p66Shc is actually recruited by RTK remain undefined.

Despite their degree of structural homology, the Shc protein isoforms are not functionally redundant. Although *ShcA*-null animals die at E11.5 (embryonic day 11.5) of development due to heart and vascularization defects [5,6], mice with *p66Shc* deficiency have a 30% increase in their average lifespan compared with control animals [7]. The latter is linked to the

Abbreviations: CH1/CH2, collagen homology domain 1/collagen homology domain 2; CSF, colony-stimulating factor 1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated kinase; Gab1, Grb2-associated binder-1; Grb2, growth factor receptor-bound protein-2; HA, haemagglutinin; HEK293, human embryonic kidney 293; HRP, horseradish peroxidase; IB, immunoblot; IP, immunoprecipitation; KD, Met kinase-dead mutant (K241A); MAPK, mitogen-activated protein kinase; Δ P, proline-rich motif p66Shc mutant (P44/47/48/50A); PI3K, phosphoinositide 3-kinase; PTB, phosphotyrosine-binding domain; Δ PTB, PTB domain inactivated (R285M); RTK, receptor tyrosine kinase; S/A, phospho-Ser-36-defective p66Shc mutant (serine to alanine); SH2, Src homology 2 domain; Δ SH2, SH2 domain inactivated (R507M); Shc, Src homology and collagen homology; TCL, total cell lysate; WT, wild-type.

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ability of the p66 isoform to promote stress-induced cell-death [7,8]. Besides, in contrast with the p52Shc isoform that promotes MAPK activation, p66Shc negatively regulates RTK-mediated activation of the MAPK pathways. In occurrence, p66Shc has been shown to inhibit MAPK activation, induced by epidermal growth factor (EGF) and insulin-like growth factor (IGF-1) stimulation, thereby reducing cell proliferation and migration [4,9–12]. The functional divergence of p66Shc among the other Shc isoforms is attributed to its unique N-terminal proline-rich CH2 domain. Although it is clear that the pro-apoptotic activity of p66Shc, in response to stress challenges, depends on Ser-36 phosphorylation, the precise mechanisms by which p66Shc negatively regulates MAPK and mitogenesis is controversial [4,9–12].

The receptor for the hepatocyte growth factor (HGF), Met, was first isolated as a constitutively active chimaeric receptor called Tpr-Met [13,14]. The signalling and biological activity of Met, like for its cytosolic oncogenic counterpart, Tpr-Met, is reliant on Met kinase activity and two critical phospho-Tyr residues within its C-terminus [15]. While Tyr-1356 (Tyr-489 in Tpr-Met) provides a direct binding site for the Grb2 and Shc adaptor proteins, Tyr-1349 (Tyr-482 in Tpr-Met) represents a direct binding site for the docking protein Grb2-associated binder-1 (Gab1) [16,17]. In addition, by virtue of its constitutive association with the Grb2 adaptor protein, Gab1 is indirectly recruited to phospho-Tyr-1356 of Met by mechanisms involving direct binding of Grb2 or indirectly through Shc [18]. In turn, Gab1 couples activated Met to multiple downstream signalling proteins including, among others, the PI3K, phospholipase C γ (PLC γ) and the SH2-containing protein tyrosine phosphatase-2 (SHP-2) [19–21]. Structure–function studies have unveiled critical roles for Shc adaptor proteins in Met-mediated biological functions. For instance, the exclusive sustained engagement of Shc-dependent signals by Met was shown to be sufficient to promote proliferation, morphological oncogenic transformation and anchorage-independent growth, as well as tumorigenesis and experimental lung metastasis in fibroblasts and intestinal epithelial cells [19–21]. Although the receptor Met can engage the three isoforms of Shc, only the structural determinants for the Met–p52Shc complex have been investigated. Thus, it remained unclear whether the additional CH2 domain of p66Shc could somehow alter its mode of interaction with the receptor Met. In the present study, this postulate was investigated by performing co-immunoprecipitation experiments following transient co-transfection with a series of Met and p66Shc mutants in human embryonic kidney 293 (HEK293) cells. Our results reveal novel non-canonical mechanisms of interaction between the receptor Met and p66Shc, and consequently of the Grb2–Gab1 complex that distinctly depend on the activation state of the receptor Met.

MATERIAL AND METHODS

Antibodies

The anti-Met polyclonal antibody, which was raised against an epitope in the C-terminal region of human Met [22,23], was kindly provided by Dr Morag Park (McGill University, Montreal, QC, Canada). The anti-pan-Shc and anti-phospho-Shc (Tyr-239/240) antibodies, recognizing p66, p52 and p46 isoforms of ShcA, were obtained from Santa Cruz Biotechnology. The anti-phospho-Ser-36 p66Shc antibody was purchased from Enzo Life Sciences and the anti-phospho-Tyr (p-Tyr-100) and anti-phospho-Met (Tyr-1234/1235) antibodies were from Cell Signaling Technology. The anti-Grb2 monoclonal and polyclonal

antibodies were purchased from BD Transduction Laboratories and Santa Cruz Biotechnology respectively. The anti-Gab1 antibody was purchased from Millipore. Anti-haemagglutinin (HA.11) monoclonal antibody was obtained from Covance, whereas the one for the detection of the β -actin was from Sigma–Aldrich Canada. Anti-mouse IgG horseradish peroxidase (HRP)-linked and Protein A HRP-linked secondary antibodies were purchased from GE Healthcare.

DNA constructs

The pcDNA3 constructs encoding the N-terminal HA-tagged p66Shc wild-type (WT) and S36A (S/A) mutant were kindly provided by Dr Yoshikuni Nagamine (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland). The replacement of Ser-36 by alanine prevents its phosphorylation. These were subcloned into the vector pLPCX (Clontech Laboratories) carrying the puromycin-resistance marker. Other p66Shc mutants were created by overlap-extension mutagenesis PCR and cloned back into pLPCX. These include p66Shc mutants harbouring a combination of Tyr to phenylalanine mutation of each Grb2-SH2 pTyr-binding motifs (nomenclature based on the mouse p66Shc amino acid sequence: Y349/350F – 2F; Tyr-423F – 1F; Y349/350/423F – 3F); a p66Shc mutant in which a putative Grb2-SH3 binding found within the CH2 domain was invalidated by the substitution of alanine for four proline residues [24] (Δ P – proline residues 44, 47, 48 and 50 replaced by alanine); and p66Shc mutants carrying pTyr-binding invalidating arginine point mutation within individual PTB or SH2 domain or both (R285M – Δ PTB; R507M – Δ SH2; R285M/R507M – 2 Δ). The pXM encoding Tpr-Met and the chimaeric colony-stimulating factor 1 (CSF)-Met receptor, and corresponding mutants were kindly provided by Dr Morag Park (McGill University, Montreal, Canada) [25,26]. The oncoprotein Tpr-Met is the result of a chromosomal rearrangement that fuses the protein dimerization motif of Tpr with the cytoplasmic domain of the Met receptor, producing a cytosolic, constitutively activated Tyr kinase [14]. The CSF-Met receptor is composed of the extracellular domain of the human CSF-1R fused to the Met transmembrane and intracellular domains [27]. The Tpr-Met C-terminal deletion mutant (Δ CT) lacking the last 45 amino acids was generated by PCR from the pXM construct. All constructs were verified by DNA sequencing.

Transient transfections in human embryonic kidney 293 cells

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wisent) containing 10 % FBS (Life Technologies) and 50 μ g/ml gentamicin (Wisent). Cell transfections were performed using FuGENE 6 or XtremeGENE HP reagents according to the manufacturer's instructions (Roche). All experiments were carried out with cells with fewer than 15 passages.

Immunoprecipitation and immunoblotting

Total cell lysate (TCL) preparation, SDS/PAGE, immunoprecipitation (IP) and immunoblot (IB) analysis methods were previously described [22]. Primary antibodies were used at a dilution of 1:1000, with the exception of the ones for Gab1 (1:500) and β -actin (1:20000). Secondary antibodies were used at a dilution of 1:10000. Proteins were visualized by enhanced chemiluminescence (GE Healthcare). Unless otherwise stated, biochemical data are representative of at least three experiments performed with independent lysate preparations of cells that had been serum-starved overnight.

RESULTS

Activated Met induces p66Shc Tyr phosphorylation

We first evaluated whether p66Shc was Tyr-phosphorylated downstream of activated Met receptor. Thus, IB was performed with an antibody detecting all Shc proteins phosphorylated on their twin-Tyr residues (P-Shc, Tyr-349/350 in p66Shc) on TCLs of HEK293 cells transiently co-transfected with a constitutively activated form of the Met receptor, Tpr-Met (or pXM vector) and the HA-tagged p66Shc protein (or pLPCX vector). Activation of Met, monitored through its Tyr-1234/1235 (P-Met), was not affected by p66Shc expression (Figure 1A). Phosphorylation of HA-p66Shc on Tyr, which migrated as a ~70 kDa protein, was revealed exclusively in Tpr-Met-expressing cells (Figure 1A). This P-Shc-specific antibody cross-reacted with phosphorylated Tpr-Met protein (65 kDa), masking the detection of endogenous p52Shc Tyr phosphorylation.

p66Shc–Met interaction is constitutive but enhanced when Met is activated

Reciprocal co-IPs were performed to determine whether Tpr-Met and p66Shc interacted. As shown in Figure 1B, Tpr-Met was recovered from HA immunoprecipitates of Tpr-Met-expressing cell extracts and Tpr-Met-induced p66Shc Tyr phosphorylation was validated (IP: HA; IB: Met and P-Shc). However, the HA-p66Shc protein was recovered by IP with an anti-human Met antibody from Tpr-Met-expressing cell lysates, but also from those transfected solely with HA-p66Shc, albeit at reduced levels (Figure 1B, IP: Met; IB: HA). Endogenous expression of non-activated Met (~145 kDa) in HEK293 cells [28] was confirmed, being detected across all samples by re-probing the membranes with a Met-specific antibody (Figure 1B, IP: Met; IB: Met), but not with an anti-pan-phospho-Tyr antibody (P-Tyr; results not shown). Recovered p66Shc protein by Met IP was Tyr-phosphorylated exclusively in Tpr-Met-expressing cells (Figure 1B, IP: Met; IB: P-Shc), but at quite low levels relative to that being detected with the same anti-P-Shc antibody by IB of TCLs (Figure 1A).

p66Shc interacts constitutively with Grb2 and Gab1, but blocks their recruitment to Met and Gab1 Tyr phosphorylation when Met is activated

We extended our analysis to evaluate Grb2 and Gab1 interaction with p66Shc and Met. Although HA-p66Shc was detected in Tpr-Met-expressing cells and at low levels in those transfected only with the HA-p66Shc construct following Grb2 IP, Tyr-phosphorylated p66Shc was only recovered in Tpr-Met-transfected cells (Figure 1B, IP: Grb2; IB: HA and P-Shc). Moreover, re-probing the blots with an anti-Met antibody showed that Tpr-Met–Grb2 interaction was nearly abolished in HA-p66Shc-expressing cells (Figure 1B, IP: Grb2; IB: Met). Analysis of Gab1-immunoprecipitates from an independent set of transfected cells (Figure 1C) showed that p66Shc binding to Gab1 was constitutive and further enhanced by Tpr-Met co-expression, and p66Shc Tyr phosphorylation coupled to Gab1 only in Tpr-Met-expressing cells (Figure 1D, IP: Gab1; IB: P-Tyr and IB: HA respectively). Reduced Gab1 Tyr phosphorylation and coupling to Tpr-Met in HA-p66Shc-expressing cells was also revealed (Figure 1D, IP: Gab1; IB: P-Tyr and Met). These results suggested constitutive p66Shc binding to Met, Grb2 and Gab1, and that p66Shc Tyr phosphorylation and recruitment to activated Met concurrently interferes with Met–Grb2–Gab1 complex assembly,

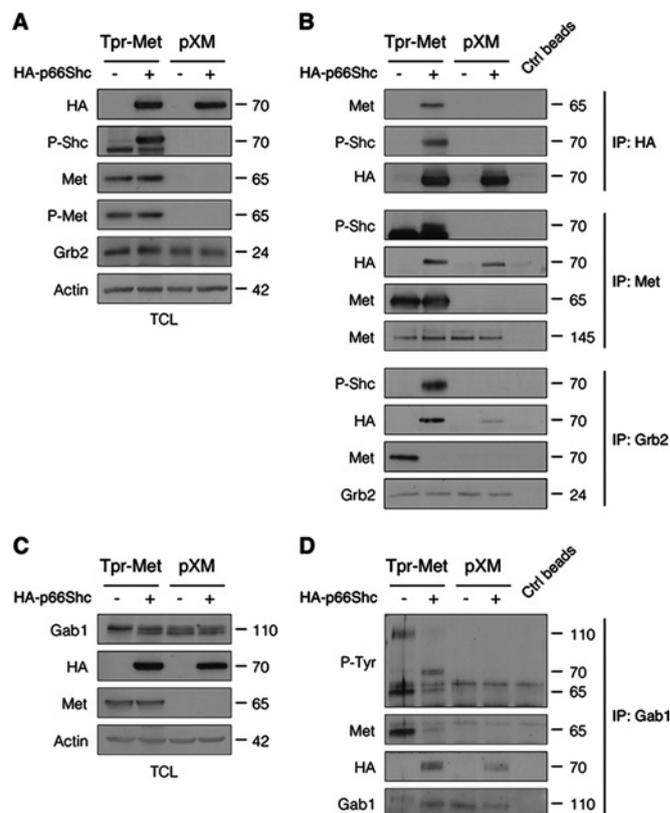


Figure 1 Non-canonical interactions of p66Shc with Met, Grb2 and Gab1

(A) Tpr-Met promotes Tyr-phosphorylation of p66Shc. HEK293 cells were transfected with Tpr-Met and/or HA-tagged p66Shc or empty vectors (pXM or pLPCX). After 48 h, cells were serum-starved overnight and TCLs were prepared. Tpr-Met and HA-p66Shc protein levels were evaluated by IB analysis. The state of Tyr phosphorylation of Tpr-Met and p66Shc proteins was assessed by IB using anti-phospho-specific Met (pY1234/35, P-Met) and Shc (pY349/350 in p66Shc, P-Shc) antibodies, respectively. β -Actin protein was probed as a loading control. (B) p66Shc binds constitutively to the endogenous receptor Met and Grb2, and inhibits the Tpr-Met–Grb2 complex. Lysates were subjected to IP with Met-, HA- or Grb2-specific antibodies, or corresponding Sepharose beads, followed by IB with the indicated antibodies. (C and D) p66Shc binds constitutively to Gab1, and blocks Gab1 Tyr phosphorylation and Tpr-Met–Gab1 interaction. (C) Protein levels of Tpr-Met, HA-p66Shc, Gab1 and actin in an independent set of transfections in HEK293 cells are shown. (D) TCLs were subjected to IP with a Gab1 antibody, which was then immunoblotted with a pan-phospho-Tyr-specific antibody (P-Tyr). The blot was stripped and re-probed for the detection of Gab1, HA-p66Shc and Tpr-Met.

while increasing p66Shc–Grb2–Gab1 interaction, which in the latter complex Gab1 is not Tyr-phosphorylated.

Met kinase activity and multisubstrate docking site are dispensable for p66Shc interaction

The mechanistic basis of the non-canonical interactions between Met, Grb2 and p66Shc was investigated by performing co-IPs on lysates of HA-p66Shc-expressing cells co-transfected with a Tpr-Met kinase-dead mutant (KD: K241A), or a mutant having Tyr-482 and Tyr-489 replaced by phenylalanine (2F). These assays were performed in parallel with WT or mutated forms of the cell-surface-localized CSF-Met receptor [27]. Both Tpr-Met-WT and -2F mutants, like the CSF-Met counterparts, were Tyr-phosphorylated but not the KD mutants (Figure 2, IB: P-Met). CSF-Met phosphorylation without ligand stimulation is consistent with Met being activated and Tyr-phosphorylated when overexpressed [29,30]. Phosphorylation of HA-p66Shc on Tyr was observed in cells expressing the Tpr-Met or

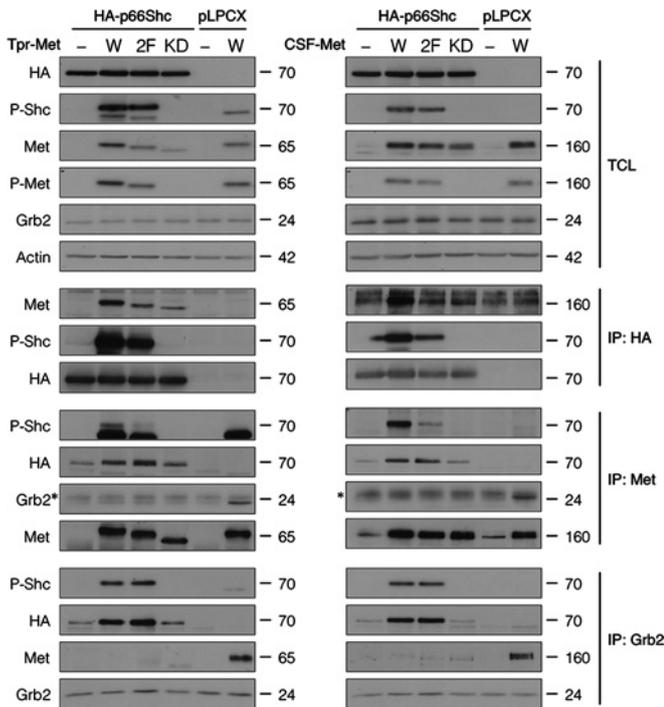


Figure 2 Met kinase activity and multisubstrate docking site are dispensable for p66Shc interaction

The indicated Tpr-Met and CSF-Met cDNA constructs, or the corresponding empty pXM vector, were co-transfected with the HA-p66Shc or the empty pLPCX vector (—). After 48 h, cells were serum-starved overnight and TCLs were prepared. The indicated IB analyses were performed to evaluate the expression and Tyr phosphorylation levels of Met receptor mutants or p66Shc proteins, and Grb2 amount. Lysates were also subjected to an IP with Met-, HA- or Grb2-specific antibodies followed by IB with the indicated antibodies. 2F denotes Tpr-Met or CSF-Met receptor mutants where both Tyr-1349 and Tyr-1356 in the C-terminal region of Met are replaced with a non-phosphorylatable phenylalanine residue. The KD corresponds to the kinase-inactive Met receptor mutants in which the ATP-binding lysine residue within the kinase domain is replaced by alanine. (*) denotes the light or heavy Ig chains of antibodies used for IP.

CSF-Met-WT and -2F constructs, but not in those expressing the KD mutants (Figure 2, IB: P-Shc). This suggests that Met-induced p66Shc Tyr phosphorylation is dependent on its kinase activity, whereas its multisubstrate-docking site is dispensable. Constitutive Met-p66Shc association was validated by reciprocal co-IPs, where p66Shc binding to Tpr-Met or CSF-Met-2F and -KD mutants was reduced but not abolished relative to unmodified Met (Figure 2, IP: HA; IB: Met and IP: Met; IB: HA). Although p66Shc Tyr phosphorylation was slightly reduced in cells expressing Met-2F mutants, when compared with cells transfected with WT counterparts (Figure 2, TCL, IB: P-Shc), the p66Shc amount in complex with Grb2 was equivalent in these cells (Figure 2, IP: Grb2; IB: HA). The pool of p66Shc protein coupled to Met-WT and -2F proteins was weakly Tyr-phosphorylated (Figure 2, IP: Met; IB: P-Shc). Significantly, Grb2 was not coupling to Tpr-Met or CSF-Met-2F mutants, as previously reported [31], but p66Shc expression severely disrupted Grb2 binding to the Met-WT counterparts (Figure 2, IP: Met; IB: Grb2 or IP: Grb2; IB: Met). Our data support that p66Shc recruitment to Met involves both pTyr-independent and -dependent mechanisms, and that the subcellular localization of Met does not have an impact on p66Shc-mediated inhibition of the Met-Grb2 interaction.

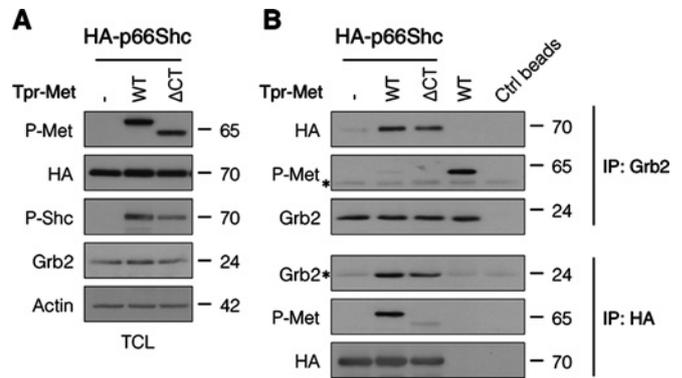


Figure 3 The C-terminal domain of Tpr-Met is not required for p66Shc recruitment and Tyr phosphorylation

(A) Tpr-Met- Δ CT mutant promotes Tyr phosphorylation of p66Shc. HEK293 cells were co-transfected with HA-p66Shc and Tpr-Met WT, or a Tpr-Met mutant deleted of its 45 C-terminal amino acids (Tpr-Met- Δ CT). After 48 h, cells were serum-starved overnight and TCLs were prepared. Cellular expression of Tpr-Met-WT and - Δ CT mutant was evaluated by IB analysis using the anti-P-Met antibody, since the Δ CT mutant protein was undetectable with all the anti-Met antibodies tested. Total and Tyr phosphorylation of p66Shc, and Grb2 and β -actin protein levels were probed. (B) p66Shc binds to the Tpr-Met- Δ CT mutant. Lysates were subjected to an IP with anti-Grb2 or -HA antibodies followed by IB with the indicated antibodies. (*) denotes the light or heavy Ig chains of antibodies used for IP.

C-terminal tail deletion in activated Met neither blocks p66Shc recruitment nor Tyr phosphorylation

Our results showed that neither Met-p66Shc interaction, nor p66Shc Tyr phosphorylation and Grb2 coupling was abolished by invalidation of the Met multisubstrate-binding site (Figure 2). Thus, we next evaluated the role of the C-terminal tail of Met by the analysis of cells co-transfected with HA-p66Shc and Tpr-Met-WT or a mutant lacking its last 45 amino acids (Tpr-Met- Δ CT). We failed to detect Tpr-Met- Δ CT mutant protein by IB with an antibody recognizing the residues surrounding Tyr-1234 (results not shown), but did confirm its expression and activation using the anti-P-Met antibody (Figure 3A, TCL, IB: P-Met). Like Tpr-Met-WT-transfected cells, those expressing the Tpr-Met- Δ CT displayed p66Shc Tyr phosphorylation (Figure 3A, TCL, IB: P-Shc), and enhanced p66Shc-Grb2 interaction (Figure 3B, IP: HA; IB: Grb2 and IP: Grb2; IB: HA). Coherent with Grb2 binding to the pTyr-489 in Tpr-Met; the Tpr-Met- Δ CT protein was not recovered from Grb2 immunoprecipitates (Figure 3B, IP: Grb2; IB: P-Met). However, p66Shc interaction with the Tpr-Met- Δ CT mutant was reduced relative to the full-length Tpr-Met protein, but not abolished (Figure 3B, IP: HA; IB: P-Met). Hence, in addition to its C-terminal tail, a distinct region within the activated Met is involved in mediating p66Shc recruitment, Tyr phosphorylation and Grb2 binding.

p66Shc PTB and SH2 domains are dispensable for p66Shc-Grb2 interaction under Met activation, whereas p66Shc PTB domain is required for its Tyr phosphorylation and Met binding, and Met-Grb2 complex inhibition

The contribution of the p66Shc PTB and SH2 domains in coordinating non-canonical interactions with Met and Grb2 was next investigated. Thus, co-IPs were performed with lysates of cells co-transfected with Tpr-Met-WT and p66Shc phospho-Tyr-binding-deficient mutants (Figure 4A, Δ PTB: R285M, Δ SH2: R507M and Δ 2D: R285/507M) [32,33]. Tpr-Met-induced p66Shc Tyr phosphorylation was unaffected by invalidation of the SH2

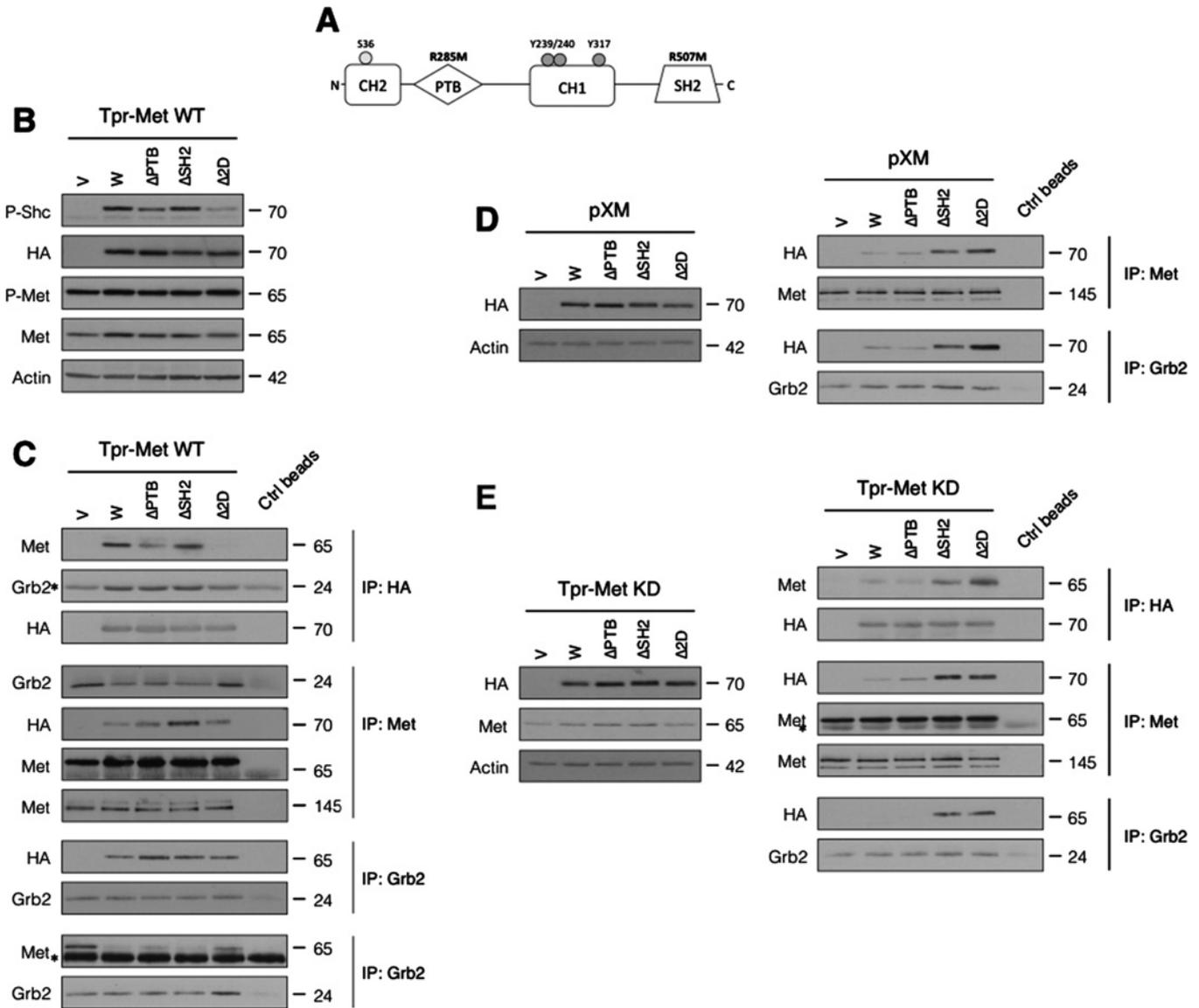


Figure 4 Dynamic inputs of the PTB or SH2 domain for Tyr phosphorylation of p66Shc, its inhibitory action on Tpr-Met-Grb2 interaction, and binding to Grb2 and non-activated Met

(A) Diagram showing p66Shc structure and mutants tested. (B) PTB and SH2 domain of p66Shc co-ordinates its Tyr phosphorylation in Tpr-Met-expressing cells. Tpr-Met cDNAs were co-transfected in HEK293 cells with either pLPCX empty vector or HA-p66Shc WT or the indicated p66Shc mutants. After 48 h, cells were serum-starved overnight and TCLs were prepared. Total cellular level of HA-p66Shc protein and state of phosphorylation on Tyr-349/350 residues, along with the cellular expression and phosphorylation levels of Tpr-Met, and β -actin protein amount were probed by IB analysis. (C) Invalidation of the PTB domain in p66Shc blocks Tpr-Met-p66Shc binding and its inhibitory action on the Tpr-Met-Grb2 complex, but the two phospho-Tyr-docking domains are dispensable for Grb2 binding. Lysates from different co-transfections were subjected to an IP with HA-, Grb2- or Met-specific antibodies followed by IB with the indicated antibodies. Notably, both the endogenous Met and exogenous Tpr-Met proteins were pulled-down in anti-Met-IP. (D and E) The SH2 domain in p66Shc negatively regulates its binding to Grb2 and inactivated Met receptor. Similar co-IP and IB analyses were carried in HEK293 cells co-transfected either with empty pXM vector (D) or kinase-inactive Met receptor mutant (E). (*) denotes the light or heavy Ig chains of antibodies used for IP.

domain, but was markedly reduced and nearly abolished when the PTB domain was disrupted alone or concurrently with the SH2 domain respectively (Figure 4B). However, reciprocal co-IPs revealed a weak loss of p66Shc-Grb2 interaction only when the PTB and SH2 domains were simultaneously inactivated (Figure 4C, IP: HA; IB: Grb2 and IP: Grb2; IB: HA). A comparable amount of Tpr-Met was recovered from HA immunoprecipitates of lysates of cells co-expressing the p66Shc-WT or - Δ SH2 mutant, but this was noticeably reduced in p66Shc- Δ PTB-transfected cells, and even more when the SH2 and PTB

domains were concurrently inactivated (Figure 4B, IP: HA; IB: Met). Moreover, Tpr-Met-Grb2 interaction was still abolished in p66Shc- Δ SH2-expressing cells, but considerably less in those transfected with PTB- or PTB/SH2-deficient p66Shc mutants (Figure 4C, IP: Met; IB: Grb2 and IP: Grb2; IB: Met). These results show that, in the context of activated Met, the p66Shc PTB and SH2 domains are dispensable for p66Shc-Grb2 interaction, whereas p66Shc Tyr phosphorylation and Met binding, like its mediated inhibition of Met-Grb2 interaction, mainly depend on the PTB domain integrity.

p66Shc SH2 domain negatively regulates its constitutive binding to Met and Grb2

Requirement of the p66Shc PTB and SH2 domains in coupling to Met was further characterized by evaluating the amount of HA-p66Shc protein recovered after Met IP. All p66Shc mutant proteins were readily detected in Met-immunoprecipitates, but the p66Shc- Δ SH2 amount recovered was more elevated than that of p66Shc-WT or other mutant proteins (Figure 4C, IP: Met; IB: HA). This suggested an inhibitory allosteric constraint imposed by the p66Shc SH2 domain in respect to its binding to inactivated Met. This was validated by co-IP analysis of lysates of cells co-transfected with p66Shc mutants and either empty pXM vector or Tpr-Met-KD mutant, where the SH2-defective p66Shc mutant showed enhanced binding to endogenous Met and Tpr-Met-KD, and even more when the PTB domain was concurrently disrupted (Figures 4D and 4E, IP: Met; IB: HA or IP: HA; IB: Met). Although not Tyr-phosphorylated, the p66Shc mutants harbouring inactivating mutations in their SH2 domain, alone or concurrently in their PTB domain, also showed increased binding to Grb2 in pXM or Tpr-Met KD co-transfected cells (Figures 4D and 4E, IP: Grb2; IB: HA). These results showed that a mechanism independent of the p66Shc PTB and SH2 domains co-ordinates p66Shc-Grb2 and p66Shc-Met constitutive interactions, where the SH2 domain exerts an allosteric inhibitory effect.

p66Shc Ser-36 phosphorylation is neither influenced by Met activation nor a critical determinant of the non-canonical Met-p66Shc-Grb2 complex formation

We next evaluated the influence of p66Shc Ser-36 phosphorylation on Met-p66Shc-Grb2 complex formation by the analysis of lysates of cells co-transfected with Tpr-Met and HA-p66Shc-WT, or a phospho-Ser-36-defective p66Shc mutant (S/A). Probing of HA immunoprecipitates with an antibody raised against this phosphorylated Ser-36 residue (anti-P-Ser-36) showed a 1.54-fold increase in p66Shc phosphorylation on Ser-36 in Tpr-Met-expressing cells relative to the pXM control cells, but which coincided with a 1.57-fold elevation in the HA-p66Shc protein level (Figure 5A, IP: HA; IB: P-Ser-36 and HA). This indicates that activation of Met signalling in HEK293 cells promotes p66Shc expression, but not its Ser-36 phosphorylation. As shown in Figure 5(B), p66Shc-WT and S/A protein phosphorylation on Tyr induced by Tpr-Met (IP: HA; IB: P-Tyr) and their binding to Tpr-Met, the endogenous Met or Grb2 were comparable (IP: HA; IB: Met; IP: Met; IB: HA and IP: Grb2; IB: HA). Notably, the HA-p66Shc-S/A mutant also markedly reduced Tpr-Met-Grb2 interaction (Figure 5B, IP: Met; IB: Grb2 and IP: Grb2; IB: Met).

The proline-rich motif in p66Shc CH2 domain inhibits constitutive binding to Met and Grb2

We next evaluated the contribution of a putative Grb2-SH3-binding site in the p66Shc CH2 domain [34]. Cells were co-transfected with Tpr-Met and a p66Shc mutant in which this putative binding was invalidated by the substitution of alanine for four proline residues [24] (Figure 6A). Analysis of HA immunoprecipitates revealed that p66Shc- Δ P Tyr phosphorylation (results not shown) and binding to Grb2 in Tpr-Met-expressing cells was similar to the p66Shc-WT, whereas its interaction with Tpr-Met was slightly more elevated (Figure 6B, IP: HA; IB: Met or IB: Grb2). Similar amounts of p66Shc- Δ P

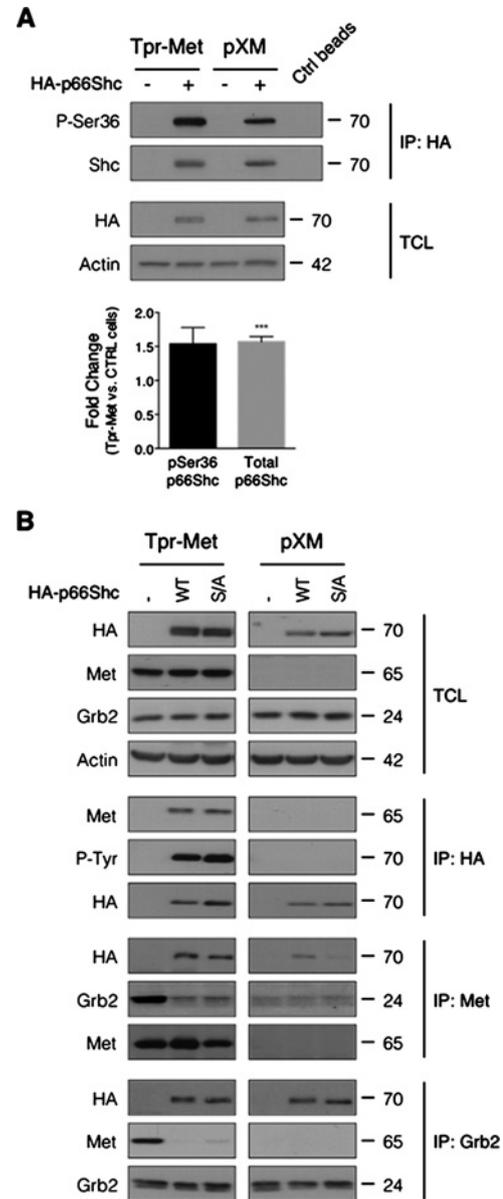


Figure 5 p66Shc binds to Met and inhibits Met-Grb2 interaction by a mechanism independent of Ser-36 phosphorylation

(A) Tpr-Met does not enhance Ser-36 phosphorylation of p66Shc. Tpr-Met or pXM vector cDNAs were co-transfected in HEK293 cells with WT HA-p66Shc or corresponding pLPCX empty vector. After 48 h, cells were serum-starved overnight and TCLs were prepared. Total cellular level of HA-p66Shc protein was determined by IB with an anti-HA antibody, whereas β -actin protein was probed as a loading control. Phosphorylation of p66Shc on Ser-36 was evaluated in HA immunoprecipitates by IB analyses with an anti-phospho-Ser-36-p66Shc antibody (P-Ser-36). The amount of HA-p66Shc protein recovered after HA-IP was estimated by re-probing the blots with anti-pan-Shc antibody. The histogram shows the fold difference (mean \pm S.D.) in Ser-36 phosphorylation and total HA-p66Shc protein levels in Tpr-Met-expressing cells relative to those detected in control cells, based on densitometric quantification. Only the increase in total HA-p66Shc level was significant based on a one-sample Student's *t* test. The cellular levels of p66Shc protein phosphorylation at Ser-36 were normalized to the amount of p66Shc protein recovered after HA IP ($n = 3$), whereas those for total HA-p66Shc protein were normalized to the level of actin ($n = 4$). (B) Interference with p66Shc Ser-36 phosphorylation does not have an impact on Met-p66Shc-Grb2 interaction. HEK293 cells were co-transfected with Tpr-Met or pXM empty vectors and either WT HA-p66Shc or the S/A mutant, in which Ser-36 was replaced by alanine (S/A). Total Tpr-Met, HA-p66Shc, Grb2 and actin protein levels in cell lysates were evaluated by IB analyses. The same cell lysates were used to carry out the indicated co-IP experiments. The slight reduced amount of p66ShcS/A relative to WT co-IP with Met was not reproducible in other experiments.

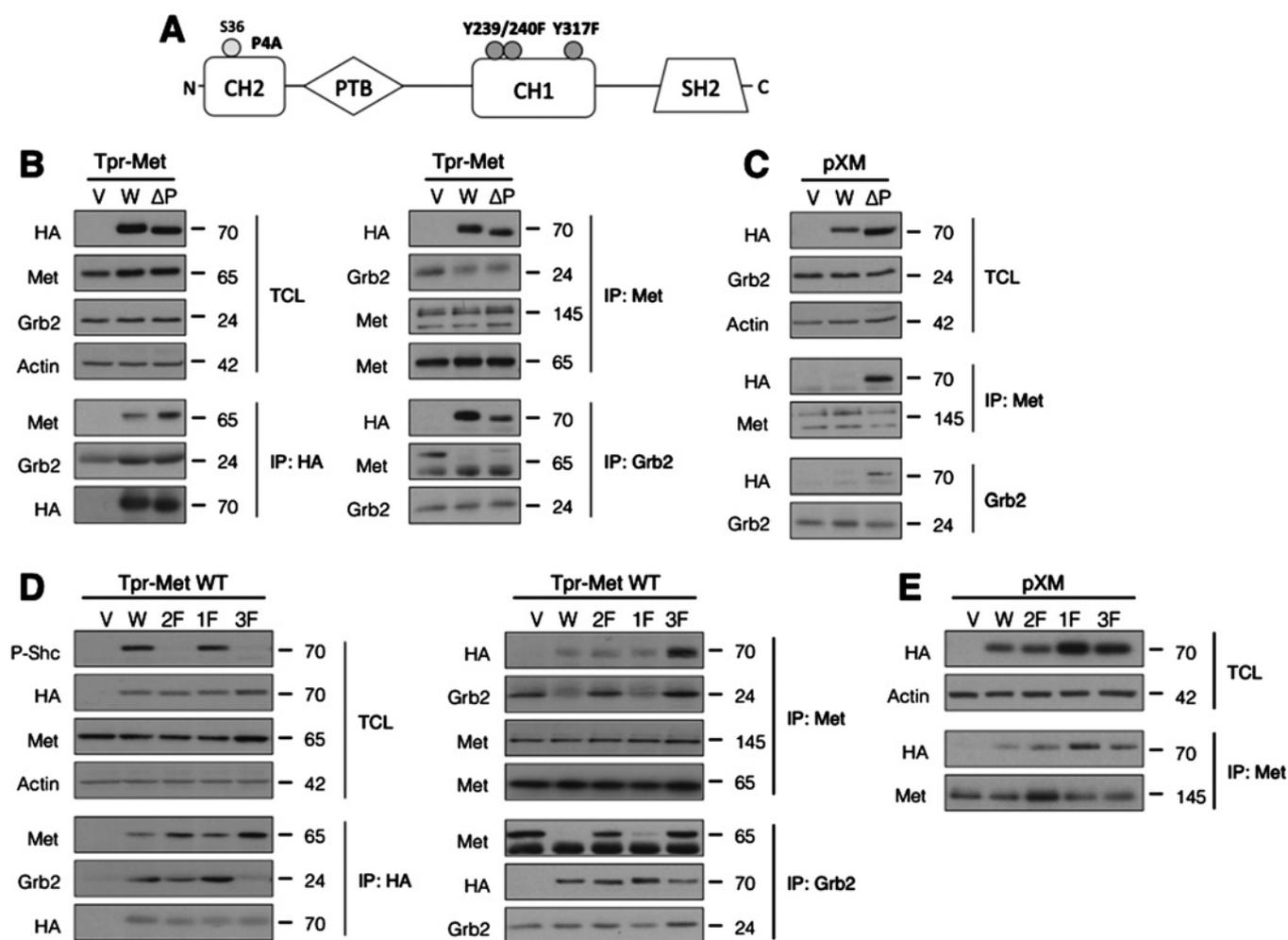


Figure 6 Phosphorylation of p66Shc on Tyr-349/350 and coupling to Grb2 are needed for inhibition of Met–Grb2 interaction

(A) Diagram showing p66Shc structure and mutants tested. (B) Invalidation of the Pro-motif in p66Shc does not block Grb2 binding and p66Shc-induced inhibition of the Tpr-Met–Grb2 complex. Tpr-Met cDNAs were co-transfected in HEK293 cells with either pLPCX empty vector or HA-p66Shc WT or the p66Shc- Δ P mutant harbouring four proline to alanine substitutions in its CH2 domain. After 48 h, cells were serum-starved overnight and TCLs were prepared. Total cellular levels of HA-p66Shc, Tpr-Met, Grb2 and β -actin protein were probed by IB analysis. Lysates from same co-transfections were subjected to an IP with anti-Met and -Grb2 antibodies, followed by IB with the indicated antibodies. (C) Invalidation of the proline-rich motif in p66Shc enhances constitutive binding to Met and Grb2. The p66Shc-WT and - Δ P mutant were co-transfected with empty pXM vector in HEK293 cells. TCLs from these co-transfections were subjected to the indicated IB and co-IP analyses. (D) Phospho-Tyr-349/350 in p66Shc binds Grb2 that blocks Tpr-Met–Grb2 interaction. The indicated p66Shc mutants harbouring Tyr to phenylalanine substitutions that invalidate Grb2-SH2 pTyr-binding sites were co-transfected with Tpr-Met in HEK293 cells. The indicated IB analyses were performed to evaluate Tyr phosphorylation and expression levels of p66Shc mutant proteins, and the expression of Tpr-Met. The level of β -actin protein was probed as a loading control. 2F, 1F and 3F denote, respectively, p66Shc mutants in which Tyr-349/350, Tyr-423 or Tyr-349/350/423 were replaced by phenylalanine. Lysates of a different set of cells co-transfected with Tpr-Met and the indicated phospho-Tyr-deficient p66Shc mutants were subjected to IP with HA-, Met- or Grb2-specific antibodies followed by IB with the indicated antibodies. N.B. both Tpr-Met and endogenous Met proteins were pulled-down upon anti-Met IP. (E) Constitutive interaction between p66Shc and endogenous Met is not affected by Tyr to phenylalanine substitutions in p66Shc. Cells were co-transfected with pXM and p66Shc Tyr to phenylalanine mutants, and the same TCLs were subjected to the indicated IB and co-IP analyses.

and p66Shc-WT were recovered from Met immunoprecipitates of lysates of Tpr-Met-expressing cells (Figure 6B, IP: Met; IB: HA). Reciprocal co-IPs showed that p66Shc-mediated inhibition of Tpr-Met–Grb2 interaction was unaffected by invalidation of its proline-rich motif (Figure 6B, IP: Met; IB: Grb2 or IP: Grb2; IB: Met). The p66Shc- Δ P mutant showed enhanced binding to both endogenous Met and Grb2 in pXM-transfected cells (Figure 6C, IP: Met; IB: HA and IP: Grb2; IB: HA). Altogether, these findings indicate that the proline-rich motif in the p66Shc CH2 domain does not play a major role in mediating its constitutive or Met-inducible interaction with Grb2. Nonetheless, when p66Shc is not Tyr-phosphorylated, this proline-rich motif hinders its binding to both the endogenous Met and Grb2.

p66Shc phospho-Tyr-349/350 residues are required for Grb2 binding and Met–Grb2 complex inhibition

The role of the phospho-Tyr motifs in the p66Shc CH1 domain was next investigated. Cells were co-transfected with Tpr-Met and p66Shc mutants harbouring combinations of Tyr to phenylalanine substitution of each Grb2-SH2 pTyr-binding motifs (Figure 6A, Y349/350F: 2F, Y423F: 1F or Y349/350/423F: 3F). Although expressed at similar levels to p66Shc-WT and -1F proteins, the p66Shc-2F and -3F mutants, which each harbours the Y349/350F substitution, were not detected in Tpr-Met-expressing cells by IB with an antibody specific for recognition of this phospho-Tyr motif in Shc (Figure 6D, IB: HA and P-Shc). Analysis of

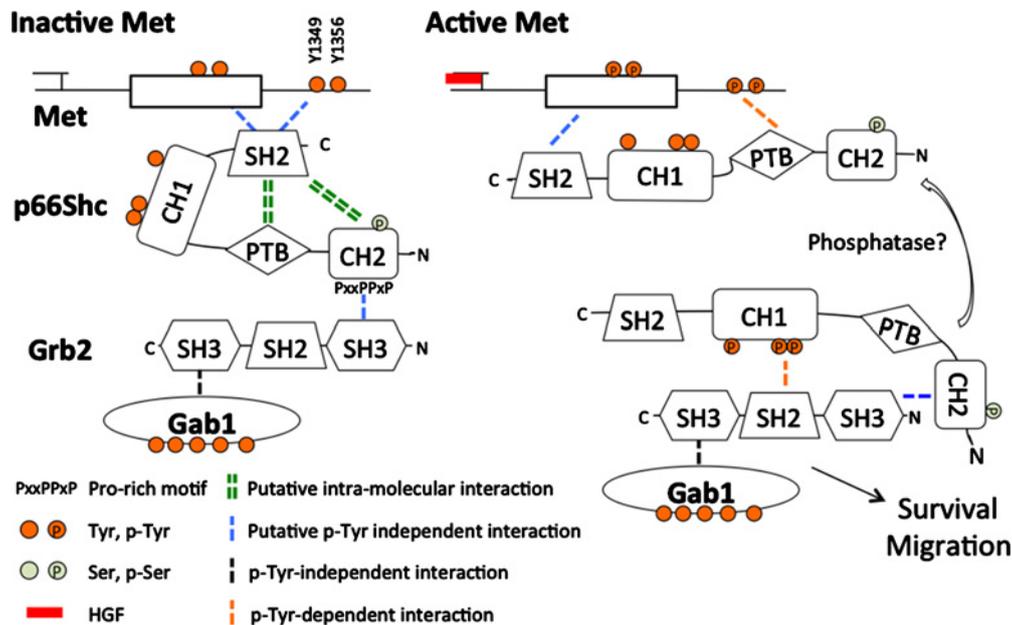


Figure 7 Proposed model for the dynamics of Met and p66Shc interactions and protein complexes

When the receptor Met is not active, the non-Tyr-phosphorylated p66Shc constitutively binds to Met, and the Grb2 and Gab1 adaptor proteins. In this instance, intramolecular constraints in p66Shc, co-ordinated by the SH2, PTB and CH2 domains, allow low-affinity interaction of p66Shc with Met, Grb2 and Gab1. When Met is activated, p66Shc becomes phosphorylated on Tyr residues, changing the core structural determinants in p66Shc dictating its interaction with Met and Grb2. Namely, the phospho-Tyr-binding integrity of the p66Shc PTB and SH2 domains is now critical for p66Shc–Met and p66Shc–Grb2–Gab1 complex formation, where the Grb2 binds to phospho-Tyr-349/350 in p66Shc. The Met multisubstrate-docking site is not required for p66Shc binding and Tyr phosphorylation, whereas it is the non-Tyr-phosphorylated p66Shc protein that preferentially binds to active Met. In turn, p66Shc–Grb2–Gab1 complex is redirected distal from the activated Met, where p66Shc is Tyr-phosphorylated but not Gab1, whereas Grb2–Gab1 recruitment to the Met receptor is inhibited.

HA immunoprecipitates of Tpr-Met-expressing cells revealed that the Y349/350F substitution enhanced p66Shc coupling to Tpr-Met, whereas reducing its interaction with Grb2 (Figure 6D, IP: HA; IB: Met or IB: Grb2). Concurrently, reciprocal co-IPs showed partial recovery of Tpr-Met–Grb2 interaction in p66Shc-2F-expressing cells (Figure 6D, IP: Met; IB: Grb2 and IP: Grb2; IB: Met). These above effects of the Y349/350F substitution in p66Shc were further potentiated when Tyr-423 was simultaneously mutated (Figure 6D, IP: HA; IB: Met p66Shc-3F). The inhibitory effect on p66Shc–Grb2 interaction by the Y349/350F substitution in p66Shc was not duplicated when the analysis of Grb2 immunoprecipitates was performed (Figure 6D, IP: Grb2; IB: HA). This discrepancy probably reflects enhanced p66Shc pull-down with the Tpr-Met–Grb2 complex, since p66Shc-2F displayed reduced capacity to block Tpr-Met/Grb2 interaction. Corroborating that activated Met binds preferentially to non-Tyr-phosphorylated p66Shc, p66Shc-3F protein was more efficiently recovered than p66ShcWT by anti-Met IPs of lysates of Tpr-Met-expressing cells, whereas the same amount of both proteins was pulled down in control cells (Figure 6D, IP: Met; IB: HA). Highest Tpr-Met–p66Shc coupling and inhibition of the p66Shc–Grb2 complex were both seen in the context of the p66Shc mutant protein in which all the three phospho-Tyr-binding sites were simultaneously invalidated. However, in pXM cells, p66Shc Tyr-deficient mutants and WT protein showed comparable coupling efficiency to Grb2 and Met (Figure 6E, IP: Met; IB: HA). These results indicate that the phospho-Tyr-349/350 motif within p66Shc constitutes the main binding site for phospho-Tyr-based interaction with Grb2 and for p66Shc-mediated inhibition of Tpr-Met–Grb2 complex.

DISCUSSION

Opposite to the p52Shc isoform, which interacts exclusively with activated RTKs, Grb2 and Gab1 through phospho-Tyr-dependent mechanisms, we show that the p66 isoform of Shc binds to Met even in the absence of receptor activation, as well as to Grb2 and non-Tyr-phosphorylated Gab1 (Figure 1). Previous studies have reported p66Shc's ability to bind epidermal growth factor receptor (EGFR) and Grb2 in the absence of receptor stimulation in various cell types [11,12,34,35]. Structural determinants co-ordinating the p66Shc–EGFR constitutive interaction remain unknown, whereas the phospho-Tyr-independent p66Shc–Grb2 association was proposed to involve binding of the Grb2 C-terminal SH3 domain to a proline-rich motif in the p66Shc CH2 region, based on *in vitro* pull-down assays using purified CH2 domain of p66Shc [34]. In sharp contrast, we show that proline-rich motif invalidation in the full-length p66Shc enhanced its coupling to endogenous Grb2 under basal state, as well as to the non-activated Met (Figure 6). This discrepancy probably reflects the loss of p66Shc intramolecular constraints in the isolated CH2 domain, which reduce Grb2–SH3–p66Shc–CH2 interaction. Likewise, p66Shc harbouring point mutations blocking the SH2 phospho-Tyr-binding capacity displayed a much-increased constitutive interaction with both Met and Grb2, and even more when its PTB domain was concurrently invalidated (Figure 4). Although typified to co-ordinate phospho-Tyr-based protein–protein interactions, the PTB and SH2 domains of p52Shc have been shown to be capable of binding non-Tyr-phosphorylated ligands. This includes coupling of the p52Shc PTB domain to protein Tyr phosphatase non-receptor type 12 (PTPN12) [36], the scaffold IQ motif-containing GTPase-activating protein 1 (IQGAP1) [37]

and extracellular-signal-regulated kinase 1 (ERK1) [38], whereas its SH2 domain constitutively binds to the Shc SH2 domain-binding protein 1 (SHCBP1) [39]. The results of the present study suggest that in the absence of Met receptor activation, each modular domain of p66Shc works in a concerted manner to make the conformation of this adaptor protein favourable for low-affinity binding to Grb2 and Met. Considering that Gab1 binds constitutively and directly to the C-terminal SH3 domains of Grb2, this suggests that p66Shc may operate as a platform to bring the Grb2–Gab1 complex in proximity to inactivated Met receptor. It is reasonable to expect that other p66Shc or Grb2 constitutive binding proteins such as the Ras nucleotide-exchange factor son of sevenless 1 (SOS1) or the small GTPase ADP-ribosylation factor 1 (ARF1) [35,40], may prove to be also constituents of the p66Shc-nucleated multiprotein complex.

Like for p52Shc [26], we demonstrate that p66Shc Tyr phosphorylation and interaction with Grb2 are enhanced when the Met receptor is activated (Figures 1 and 2). In this instance, the core structural elements co-ordinating p66Shc binding to active Met and Grb2 somewhat follow the p52Shc-like canonical model. Namely, the phospho-Tyr-binding integrity of p66Shc PTB domain, and to some extent of its SH2 domain, is critical for the assembly of p66Shc–Met and phospho-Tyr-based p66Shc–Grb2 complexes (Figure 4), where phospho-Tyr-349/350 in p66Shc are critical for Met-inducible p66Shc–Grb2 association (Figure 6). In sharp contrast, p66Shc binding to activated Met and p66Shc Tyr phosphorylation are not abolished by invalidation of the Met multisubstrate-docking site or by deletion of its C-terminal tail (Figures 2 and 3). Besides, p66Shc severely blocks Grb2 and Gab1 coupling to active Met, as well as Gab1 Tyr phosphorylation (Figures 1 and 2). The inhibitory action of p66Shc in respect to Met–Grb2 interaction is surprising. Although the NATpY1356VNV motif found in the Met multisubstrate-binding site constitutes a high affinity Grb2-SH2-binding motif [41], it allows feeble interaction with the Shc-PTB domain, since it is not an optimal consensus PTB-binding site, having an alanine residue, instead of proline, at position –2 of the phospho-Tyr [26]. It is tempting to speculate that p66Shc may adopt a different conformation when Tyr is phosphorylated, allowing its binding to the phospho-Tyr-1356 of Met via its PTB and to another region situated outside the Met C-terminal region, ultimately sterically hindering Grb2 and Gab1 recruitment to activated Met. Yet, our data imply that it is the non-Tyr-phosphorylated p66Shc protein species that binds predominantly to active Met, instead of the Tyr-phosphorylated forms (Figures 1 and 6). Additional studies are required to map the residues specifying Met–p66Shc interaction, as well as the mechanisms by which p66Shc inhibits Grb2 and Gab1 recruitment to activated Met. Nonetheless, our study provides critical novel insights into the p66Shc–Met interaction mode, and uncover that Tyr phosphorylation of the p66Shc protein extends beyond generating recognition motifs for effectors, altering the PTB, SH2 and CH2 domains, target-binding affinity and selectivity.

A number of studies underscore that pre-assembly of specific protein complexes to non-stimulated RTKs does not merely reflect non-specific interactions, but actually represents a regulatory mechanism of RTK basal activity and signalling of physiological relevance [42]. To this effect, the ShcD adaptor protein, which shares the same CH2-PTB-CH1-SH2 domain organization of p66Shc [43,44], has been shown to associate with ligand-free EGFR, promoting phosphorylation of specific EGFR Tyr residues (Tyr-1068/1148/1173) [45]. Ligand-independent ShcD-driven EGFR binding and phosphorylation was revealed to promote cell migration without activation of EGFR kinase or downstream MAPK/ERK and PI3K/Akt pathways [45]. Likewise,

p66Shc overexpression in human MDA-231 and SKBR3 breast cancer cells was reported to enhance migration in the absence of EGF stimulation [35]. Noteworthy, p66Shc overexpression was revealed in the ErbB2-driven breast cancer model to promote, *in cellulo* and *in vivo*, epithelial–mesenchymal transition and cell migration by a mechanism dependent on Met activation [46]. In the present study, p66Shc–Met complex formation did not concur with inducible phosphorylation of endogenous Met (Figure 1) but perhaps the p66Shc-induced Met phosphorylation level was below the limits of detection within our setting or is cell-type-specific.

Collectively, our results support a model whereby p66Shc constitutively binds to Met, Grb2 and Gab1 (Figure 7). When Met is inactive, thus p66Shc is not Tyr-phosphorylated, the SH2, PTB and CH2 domains of p66Shc impose intramolecular constraints maintaining low-affinity p66Shc–Met and p66Shc–Grb2–Gab1 interactions. Upon Met receptor activation, Tyr phosphorylation of p66Shc works as a switch, altering the core structural determinants directing p66Shc binding to Met and Grb2. Thereby, p66Shc–Met and phospho-Tyr-based p66Shc–Grb2–Gab1 complex formation becomes dependent on the phospho-Tyr-binding integrity of the p66Shc PTB and SH2 domains. However, the Met multisubstrate-docking site is not required for p66Shc binding and Tyr phosphorylation, and it is mainly the non-Tyr-phosphorylated p66Shc protein that binds to activated Met. This, in turn, redirects the p66Shc–Grb2–Gab1 complex away from the activated Met where p66Shc is Tyr-phosphorylated but not Gab1, and concomitantly prevents Grb2–Gab1 complex from contacting the Met receptor.

In summary, the present study underscores that the additional CH2 domain in p66Shc imposes unique diversification relative to its cognate p52Shc protein, in respect to its interaction mode with the receptor Met and its effects on rewiring binding effector complexes. Notably, we provide novel insights into the mechanistic basis governing dynamic non-canonical Met–p66Shc–Grb2–Gab1 protein interactions that are dependent on the activation state of the receptor, expected to be of critical relevance in achieving signal integration and function specificity in cells.

AUTHOR CONTRIBUTION

Mélissa Landry contributed to the conception of the study, carried out the initial molecular studies and generated most of the p66Shc mutants. Véronique Pomerleau performed molecular studies and drafted the paper. Caroline Saucier co-ordinated all aspects of the study and participated in writing the paper.

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REFERENCES

- Lemmon, M.A. and Schlessinger, J. (2010) Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117–1134 [CrossRef PubMed](#)
- Wills, M.K. and Jones, N. (2012) Teaching an old dogma new tricks: twenty years of Shc adaptor signalling. *Biochem. J.* **447**, 1–16 [CrossRef PubMed](#)

- 3 Ventura, A., Luzzi, L., Pacini, S., Baldari, C.T. and Pelicci, P.G. (2002) The p66Shc longevity gene is silenced through epigenetic modifications of an alternative promoter. *J. Biol. Chem.* **277**, 22370–22376 [CrossRef PubMed](#)
- 4 Migliaccio, E., Mele, S., Salcini, A.E., Pelicci, G., Lai, K.M., Superti-Furga, G., Pawson, T., Di Fiore, P.P., Lanfrancone, L. and Pelicci, P.G. (1997) Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. *EMBO J.* **16**, 706–716 [CrossRef PubMed](#)
- 5 Lai, V.K.-M. and Pawson, T. (2000) The ShcA phosphotyrosine docking protein sensitizes cardiovascular signaling in the mouse embryo. *Genes Dev.* **14**, 1132–1145 [PubMed](#)
- 6 Hardy, W.R., Li, L., Wang, Z., Sedy, J., Fawcett, J., Frank, E., Kucera, J. and Pawson, T. (2007) Combinatorial ShcA docking interactions support diversity in tissue morphogenesis. *Science* **317**, 251–256 [CrossRef PubMed](#)
- 7 Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P.P., Lanfrancone, L. and Pelicci, P.G. (1999) The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature* **402**, 309–313 [CrossRef PubMed](#)
- 8 Ma, Z., Myers, D.P., Wu, R.F., Nwariaku, F.E. and Terada, L.S. (2007) p66Shc mediates anoikis through RhoA. *J. Cell Biol.* **179**, 23–31 [CrossRef PubMed](#)
- 9 Kisielow, M., Kleiner, S., Nagasawa, M., Faisal, A. and Nagamine, Y. (2002) Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA. *Biochem. J.* **363**, 1–5 [CrossRef PubMed](#)
- 10 Natalicchio, A., Laviola, L., De Tullio, C., Renna, L.A., Montrone, C., Perrini, S., Valenti, G., Prociro, G., Svelto, M. and Giorgino, F. (2004) Role of the p66Shc isoform in insulin-like growth factor I receptor signaling through MEK/Erk and regulation of actin cytoskeleton in rat myoblasts. *J. Biol. Chem.* **279**, 43900–43909 [CrossRef PubMed](#)
- 11 Okada, S., Kao, A.W., Ceresa, B.P., Blaikie, P., Margolis, B. and Pessin, J.E. (1997) The 66-kDa Shc isoform is a negative regulator of the epidermal growth factor-stimulated mitogen-activated protein kinase pathway. *J. Biol. Chem.* **272**, 28042–28049 [CrossRef PubMed](#)
- 12 Xi, G., Shen, X. and Clemmons, D.R. (2008) p66shc negatively regulates insulin-like growth factor I signal transduction via inhibition of p52shc binding to Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 leading to impaired growth factor receptor-bound protein-2 membrane recruitment. *Mol. Endocrinol.* **22**, 2162–2175 [CrossRef PubMed](#)
- 13 Cooper, C.S., Park, M., Blair, D.G., Tainsky, M.A., Huebner, K., Croce, C.M. and Vande Woude, G.F. (1984) Molecular cloning of a new transforming gene from a chemically transformed human cell line. *Nature* **311**, 29–33 [CrossRef PubMed](#)
- 14 Park, M., Dean, M., Cooper, C.S., Schmidt, M., O'Brien, S.J., Blair, D.G. and Vande Woude, G.F. (1986) Mechanism of met oncogene activation. *Cell* **45**, 895–904 [CrossRef PubMed](#)
- 15 Peschard, P. and Park, M. (2007) From Tpr-Met to Met, tumorigenesis and tubes. *Oncogene* **26**, 1276–1285 [CrossRef PubMed](#)
- 16 Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G. and Comoglio, P.M. (1994) A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell* **77**, 261–271 [CrossRef PubMed](#)
- 17 Fixman, E.D., Fournier, T.M., Kamikura, D.M., Naujokas, M.A. and Park, M. (1996) Pathways downstream of Shc and Grb2 are required for cell transformation by the tpr-Met oncoprotein. *J. Biol. Chem.* **271**, 13116–13122 [CrossRef PubMed](#)
- 18 Lock, L.S., Frigault, M.M., Saucier, C. and Park, M. (2003) Grb2-independent recruitment of Gab1 requires the C-terminal lobe and structural integrity of the Met receptor kinase domain. *J. Biol. Chem.* **278**, 30083–30090 [CrossRef PubMed](#)
- 19 Fixman, E.D., Holgado-Madruga, M., Nguyen, L., Kamikura, D.M., Fournier, T.M., Wong, A.J. and Park, M. (1997) Efficient cellular transformation by the Met oncoprotein requires a functional Grb2 binding site and correlates with phosphorylation of the Grb2-associated proteins, Cbl and Gab1. *J. Biol. Chem.* **272**, 20167–20172 [CrossRef PubMed](#)
- 20 Maroun, C.R., Holgado-Madruga, M., Royal, I., Naujokas, M.A., Fournier, T.M., Wong, A.J. and Park, M. (1999) The Gab1 PH domain is required for localization of Gab1 at sites of cell–cell contact and epithelial morphogenesis downstream from the met receptor tyrosine kinase. *Mol. Cell. Biol.* **19**, 1784–1799 [CrossRef PubMed](#)
- 21 Weidner, K.M., Di Cesare, S., Sachs, M., Brinkmann, V., Behrens, J. and Birchmeier, W. (1996) Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. *Nature* **384**, 173–176 [CrossRef PubMed](#)
- 22 Bernier, J., Chhababi, W., Pomerleau, V. and Saucier, C. (2010) Oncogenic engagement of the Met receptor is sufficient to evoke angiogenic, tumorigenic, and metastatic activities in rat intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **299**, G677–686 [CrossRef PubMed](#)
- 23 Pomerleau, V., Landry, M., Bernier, J., Vachon, P.H. and Saucier, C. (2014) Met receptor-induced Grb2 or Shc signals both promote transformation of intestinal epithelial cells, albeit they are required for distinct oncogenic functions. *BMC Cancer* **14**, 240 [CrossRef PubMed](#)
- 24 Lock, L.S., Royal, I., Naujokas, M.A. and Park, M. (2000) Identification of an Atypical Grb2 carboxyl-terminal SH3 domain binding site in gab docking proteins reveals Grb2-dependent and -independent recruitment of Gab1 to receptor tyrosine kinases. *J. Biol. Chem.* **275**, 31536–31545 [CrossRef PubMed](#)
- 25 Rodrigues, G.A. and Park, M. (1993) Dimerization mediated through a leucine zipper activates the oncogenic potential of the met receptor tyrosine kinase. *Mol. Cell. Biol.* **13**, 6711–6722 [CrossRef PubMed](#)
- 26 Saucier, C., Papavasiliou, V., Palazzo, A., Naujokas, M.A., Kremer, R. and Park, M. (2002) Use of signal specific receptor tyrosine kinase oncoproteins reveals that pathways downstream from Grb2 or Shc are sufficient for cell transformation and metastasis. *Oncogene* **21**, 1800–1811 [CrossRef PubMed](#)
- 27 Zhu, H., Naujokas, M.A. and Park, M. (1994) Receptor chimeras indicate that the met tyrosine kinase mediates the motility and morphogenic responses of hepatocyte growth/scatter factor. *Cell Growth Differ.* **5**, 359–366 [PubMed](#)
- 28 Sakkab, D., Lewitzky, M., Posern, G., Schaeper, U., Sachs, M., Birchmeier, W. and Feller, S.M. (2000) Signaling of hepatocyte growth factor/scatter factor (HGF) to the small GTPase Rap1 via the large docking protein Gab1 and the adapter protein CRKL. *J. Biol. Chem.* **275**, 10772–10778 [CrossRef PubMed](#)
- 29 Mai, A., Muharram, G., Barrow-McGee, R., Baghiro, H., Rantala, J., Kermorgant, S. and Ivaska, J. (2014) Distinct c-Met activation mechanisms induce cell rounding or invasion through pathways involving integrins, RhoA and HIP1. *J. Cell Sci.* **127**, 1938–1952 [CrossRef PubMed](#)
- 30 Lutterbach, B., Zeng, Q., Davis, L.J., Hatch, H., Hang, G., Kohl, N.E., Gibbs, J.B. and Pan, B.S. (2007) Lung cancer cell lines harboring MET gene amplification are dependent on Met for growth and survival. *Cancer Res.* **67**, 2081–2088 [CrossRef PubMed](#)
- 31 Kamikura, D.M., Naujokas, M.A. and Park, M. (1996) Identification of tyrosine 489 in the carboxy terminus of the Tpr-Met oncoprotein as a major site of autophosphorylation. *Biochemistry* **35**, 1010–1017 [CrossRef PubMed](#)
- 32 van der Geer, P., Wiley, S., Gish, G.D., Lai, V.K., Stephens, R., White, M.F., Kaplan, D. and Pawson, T. (1996) Identification of residues that control specific binding of the Shc phosphotyrosine-binding domain to phosphotyrosine sites. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 963–968 [CrossRef PubMed](#)
- 33 Velazquez, L., Gish, G.D., van Der Geer, P., Taylor, L., Shulman, J. and Pawson, T. (2000) The shc adaptor protein forms interdependent phosphotyrosine-mediated protein complexes in mast cells stimulated with interleukin 3. *Blood* **96**, 132–138 [PubMed](#)
- 34 Khanday, F.A., Santhanam, L., Kasuno, K., Yamamori, T., Naqvi, A., Derico, J., Bugayenko, A., Mattagajasingh, I., Disanza, A., Scita, G. and Irani, K. (2006) Sos-mediated activation of rac1 by p66shc. *J. Cell Biol.* **172**, 817–822 [CrossRef PubMed](#)
- 35 Haines, E., Saucier, C. and Claing, A. (2014) The adaptor proteins p66Shc and Grb2 regulate the activation of the GTPases ARF1 and ARF6 in invasive breast cancer cells. *J. Biol. Chem.* **289**, 5687–5703 [CrossRef PubMed](#)
- 36 Charest, A., Wagner, J., Jacob, S., McGlade, C.J. and Tremblay, M.L. (1996) Phosphotyrosine-independent binding of SHC to the NPLH sequence of murine protein-tyrosine phosphatase-PEST. Evidence for extended phosphotyrosine binding/phosphotyrosine interaction domain recognition specificity. *J. Biol. Chem.* **271**, 8424–8429 [CrossRef PubMed](#)
- 37 Smith, M.J., Hardy, W.R., Li, G.Y., Goudreau, M., Hersch, S., Metalnikov, P., Starostine, A., Pawson, T. and Ikura, M. (2010) The PTB domain of ShcA couples receptor activation to the cytoskeletal regulator IQGAP1. *EMBO J.* **29**, 884–896 [CrossRef PubMed](#)
- 38 Suen, K.M., Lin, C.C., George, R., Melo, F.A., Biggs, E.R., Ahmed, Z., Drake, M.N., Arur, S., Arold, S.T. and Ladbury, J.E. (2013) Interaction with Shc prevents aberrant Erk activation in the absence of extracellular stimuli. *Nat. Struct. Mol. Biol.* **20**, 620–627 [CrossRef PubMed](#)
- 39 Schmandt, R., Liu, S.K. and McGlade, C.J. (1999) Cloning and characterization of mPAL, a novel Shc SH2 domain-binding protein expressed in proliferating cells. *Oncogene* **18**, 1867–1879 [CrossRef PubMed](#)
- 40 McDonald, C.B., El Hokayem, J., Zafar, N., Balke, J.E., Bhat, V., Mikles, D.C., Deegan, B.J., Seldene, K.L. and Farooq, A. (2013) Allosteric mediates ligand binding to Grb2 adaptor in a mutually exclusive manner. *J. Mol. Recognit.* **26**, 92–103 [CrossRef PubMed](#)
- 41 Cussac, D., Frech, M. and Chardin, P. (1994) Binding of the Grb2 SH2 domain to phosphotyrosine motifs does not change the affinity of its SH3 domains for Sos proline-rich motifs. *EMBO J.* **13**, 4011–4021 [PubMed](#)
- 42 Belov, A.A. and Mohammadi, M. (2012) Grb2, a double-edged sword of receptor tyrosine kinase signaling. *Sci. Signal.* **5**, pe49 [CrossRef PubMed](#)

-
- 43 Fagiani, E., Giardina, G., Luzi, L., Cesaroni, M., Quarto, M., Capra, M., Germano, G., Bono, M., Capillo, M., Pelicci, P. and Lanfrancone, L. (2007) RaLP, a new member of the Src homology and collagen family, regulates cell migration and tumor growth of metastatic melanomas. *Cancer Res.* **67**, 3064–3073 [CrossRef PubMed](#)
- 44 Jones, N., Hardy, W.R., Friese, M.B., Jorgensen, C., Smith, M.J., Woody, N.M., Burden, S.J. and Pawson, T. (2007) Analysis of a Shc family adaptor protein, ShcD/Shc4, that associates with muscle-specific kinase. *Mol. Cell. Biol.* **27**, 4759–4773 [CrossRef PubMed](#)
- 45 Wills, M.K., Tong, J., Tremblay, S.L., Moran, M.F. and Jones, N. (2014) The ShcD signaling adaptor facilitates ligand-independent phosphorylation of the EGF receptor. *Mol. Biol. Cell.* **25**, 739–752 [CrossRef PubMed](#)
- 46 Hudson, J., Ha, J.R., Sabourin, V., Ahn, R., La Selva, R., Livingstone, J., Podmore, L., Knight, J., Forrest, L., Beauchemin, N. et al. (2014) p66ShcA promotes breast cancer plasticity by inducing an epithelial-to-mesenchymal transition. *Mol. Cell. Biol.* **34**, 3689–3701 [CrossRef PubMed](#)
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