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Individual Src-family tyrosine kinases direct the degradation or protection of the clock protein Timeless via differential ubiquitylation

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Abstract

Timeless was originally identified in *Drosophila* as an essential component of circadian cycle regulation, where its function is tightly controlled at the protein level by tyrosine phosphorylation and subsequent degradation. In mammals, Timeless has also been implicated in circadian rhythms as well as cell cycle control and embryonic development. Here we report that mammalian Timeless is an SH3 domain-binding protein and substrate for several members of the Src protein-tyrosine kinase family, including Fyn, Hck, c-Src and c-Yes. Co-expression of Tim with Fyn or Hck was followed by ubiquitylation and subsequent degradation in human 293T cells. While c-Src and c-Yes also promoted Tim ubiquitylation, in this case ubiquitylation correlated with Tim protein accumulation rather than degradation. Both c-Src and c-Yes selectively promoted modification of Tim through Lys63-linked polyubiquitin, which may explain the differential effects on Tim protein turnover. These data show distinct and opposing roles for individual Src-family members in the regulation of Tim protein levels, suggesting a unique mechanism for the regulation of Tim function in mammals.

Keywords

Timeless; Ubiquitylation; Clock proteins; Src-family Kinases

1. Introduction

All organisms from cyanobacteria to mammals have an internal timekeeping system, the circadian clock, which imposes a 24-hour cycle regulating essential functions of metabolism and behavior [1, 2]. The circadian clock involves an autofeedback loop composed of cycling gene products that control their own expression. The *Drosophila* circadian clock is one of the best studied to date. In this system, the proteins Clock and Cycle activate transcription of Period (Per) and Timeless (Tim) which in turn suppress Clock and Cycle. Precisely timed negative feedback by Tim and Per results in rhythmic transcription of the Clock and Cycle genes [3]. Regulated degradation of the Tim and Per proteins allows the Clock and Cycle RNA levels to rise again. Thus, a key aspect of the circadian cycle is the tight regulation of

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Tim and Per protein stability [4, 5]. In *Drosophila*, the circadian clock is reset by the light-dependent degradation of the Tim protein [6–9]. Degradation of the fly Tim protein is preceded by tyrosine phosphorylation, although the identity of the tyrosine kinase responsible for this key regulatory phosphorylation event is not clear [8].

The mammalian clock is more complex than in the fly, with circadian rhythm under the control of the suprachiasmatic nucleus in the brain [10]. Although Tim has not been definitively implicated in the mammalian circadian clock [11], it associates with Per and exhibits a 24-hour oscillation of protein levels [1, 12]. Mammalian Tim plays additional roles in cell cycle regulation and may connect the circadian cycle and DNA damage checkpoints [13, 14]. High levels of Tim expression has been associated with advanced colorectal cancer [15] and Tim promoter hypomethylation correlates with increased breast cancer risk [16], underscoring the importance of Tim regulation in physiological control of the cell cycle.

Genetic studies show that mammalian Tim has an essential role in early embryonic development. Homozygous knockout of Tim produces embryonic lethality in mice. Tim-null embryos fail to develop past ED 7.5 and lack cellular organization, with necrotic cell debris filling the amniotic cavity [11]. Recent work from our group has pinpointed a function for Tim in developmental apoptosis. Embryoid bodies formed from Tim-knockdown embryonic stem (ES) cells failed to cavitate, supporting a role for Tim in triggering apoptosis to clear the proamniotic cavity [17]. Cells retained within the centers of the failed cavities continued to express the pluripotency marker Oct4, suggesting that further development is arrested without Tim. In the embryonic kidney, Tim expression is high in regions of active ureteric bud branching. Downregulation of Tim resulted in profound inhibition of embryonic kidney growth and ureteric bud morphogenesis, suggesting a role for Tim in organogenesis as well [18].

Studies summarized above indicate that Tim serves diverse biological roles in mammalian cells, and strict control of Tim protein levels may be essential for regulation of these functions as in the control of circadian rhythms. In this study, we show for the first time that Tim is a substrate for several members of the Src protein-tyrosine kinase family. Src-family kinases (SFKs) regulate multiple functions in almost every cell type, including proliferation, survival, motility and differentiation [19–21]. Our data show that SFKs recognize Tim via their SH3 domains, leading to Tim phosphorylation and subsequent ubiquitylation. Surprisingly, individual Src-family members induced Tim ubiquitylation on distinct lysine sites (K48 vs. K63), leading to either protein degradation or accumulation. Our data suggest that individual Src-family members have distinct roles in the control of Tim protein levels, providing a novel mechanism for tight control of Tim function.

2. Materials and Methods

2.1. Antibodies

The antibodies used in this study were purchased from the following vendors: V5 epitope tag (AB37292) and actin (MAB1501), Chemicon; phosphotyrosine (pY99; sc-7020), Fyn (sc-16) and Hck (sc-72), Santa Cruz Biotechnology; Src activation loop (pY416; 05–677) and ubiquitin (04–454), Millipore; c-Src (#2125) and the K63-specific ubiquitin (#5621), Cell Signaling Technologies; c-Yes (ab13954), Abcam.

2.2. Expression plasmids

The SH3 domains of murine Fyn, Hck, c-Src and c-Yes were amplified by PCR and subcloned into the bacterial expression vector pGEX-2T (GE Healthcare Life Sciences) in-frame with the GST coding region. Site-directed mutagenesis (QuikChange II XL System,

Stratagene) was used to substitute the codon for the conserved tryptophan residue on the binding surface of each SH3 domain with alanine for use as a negative control. A full-length mouse c-Yes cDNA in the mammalian expression vector pCMV-Sport6 was purchased from Open Biosystems. Expression vectors for full-length mouse Fyn, Hck and c-Src cDNAs are described elsewhere [22]. Kinase-dead (KD) mutants of Hck (K269D) [23] and c-Src (K298D), were created by site-directed mutagenesis and subcloned into pcDNA3.1. A V5-tagged Timeless cDNA in the mammalian expression vector pcDNA3.1 (Tim-V5) was the gift of Dr. Steven M. Reppert, University of Massachusetts Medical School [11]. Lys63- and Lys48-specific ubiquitin cDNA clones (in which all other lysines are replaced with arginine) were purchased from Addgene in the mammalian expression vector pRK5-HA.

2.3. Cell culture

Culture and transfection of 293T cells were performed as described [24].

2.4. SH3 binding assay

The SFK GST-SH3 domain fusion proteins and corresponding tryptophan to alanine mutants were expressed in *E. coli* and immobilized on glutathione-agarose beads (Sigma) as described elsewhere [24, 25]. Soluble protein extracts from 293T cells expressing Timeless-V5 were prepared in lysis buffer [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 2 mM NaF, 2 mM sodium orthovanadate, Protease Inhibitor Cocktail III (Calbiochem), and Benzonase (Novagen)] and incubated with the immobilized wild-type or mutant GST-SH3 fusion proteins (50 μg) in a final volume of 1 ml. Precipitated protein complexes were washed three times with 1.5 ml lysis buffer and associated proteins were resolved by 10% SDS-PAGE, transferred to PVDF membranes, and immunoblotted for Timeless with the V5 antibody. Where indicated, quantitative immunoblotting was performed by probing the transfer membranes with IRdye680 and IRdye800CW-conjugated secondary antibodies (LI-COR), followed by scanning with the Odyssey infrared imaging system (Li-COR). Protein band intensities were quantified with the Odyssey software as described elsewhere [26]. Immunoblots developed with NBT/BCIP were scanned and bands quantified with Image J [27]. Additional details of immunoblotting and immunoprecipitation procedures are described elsewhere [24].

3. Results

3.1. Domain organization of Tim and interaction with SFK SH3 domains

The Tim protein consists of two large conserved regions (Timeless and Timeless C) as well as three putative DNA-binding motifs XAP5, DDT and M/S (Figure 1A). While XAP5 domains are often associated with nuclear proteins and may confer DNA binding activity, the specific function of Tim XAP5 is unknown [28]. Mutation of the *Arabidopsis* circadian timekeeper XAP5 motif impaired regulation of the circadian clock and photomorphogenesis [29], suggesting a possible function for the XAP5 domain in circadian cycle control. The DDT (DNA binding homeobox and Different Transcription factors) domain is associated with a number of transcription and chromatin remodeling factors [30]. The M/S (Myb/SANT) region defines another nuclear DNA binding motif, and belongs to the SANT domain family [31]. The presence of these three putative DNA binding motifs is consistent with the association of Tim with the replication fork complex [32] and strengthens the idea that Tim may also function as a transcription factor [18].

Turnover of *Drosophila* Tim is preceded by tyrosine phosphorylation, although the identity of the protein-tyrosine kinase responsible for Tim phosphorylation has not been reported (see Introduction). Our previous work identified Tim as a c-Src SH3 domain binding protein and substrate in ES cells [17], although the effect of Src-mediated tyrosine phosphorylation

on Tim function was not established. In order to determine whether this interaction was unique to c-Src, we expanded our study to include additional members of the Src kinase family which have also been implicated in ES cell growth and differentiation (Hck, c-Yes, and c-Src). In this cellular context, Hck and c-Yes may function in self-renewal while c-Src promotes differentiation [22, 33].

To determine whether SH3 domains derived from Src-family members other than c-Src also associated with Tim, recombinant SH3 domains from Fyn, Hck, c-Yes as well as c-Src were immobilized on glutathione-agarose beads and incubated with lysates from 293T cells expressing epitope-tagged Tim. As a negative control, binding reactions were run in parallel with inactive SH3 domains in which the conserved tryptophan residue on the SH3-binding surface is replaced with alanine [24]. As shown in Figure 1B, Tim interacted with the SH3 domains of Hck, c-Src and c-Yes in this assay, while relatively weak interaction was observed with the SH3 domain of Fyn. Tim binding was not observed with any of the mutant SH3 domains or to GST alone, supporting a specific SH3-mediated interaction. These data suggest that Tim has the potential to interact with several SFKs through their SH3 domains.

3.2. Tim is a SFK substrate

We next investigated whether Tim is a SFK substrate by co-expressing it with full-length Fyn, Hck, c-Src and c-Yes in 293T cells. Tim was immunoprecipitated from transfected cell lysates via a C-terminal V5 epitope tag, followed by anti-phosphotyrosine immunblotting. As shown in Figure 2, Tim was phosphorylated by both Hck and c-Src, and to a lesser extent by Fyn and c-Yes. Cell lysates were also blotted with phosphospecific antibodies that recognize the tyrosine-phosphorylated activation loop of each SFK. A strong signal was detected in each case, demonstrating that the transfected SFKs were active. These results show that Tim is a substrate for multiple members of the Src kinase family.

As tyrosine phosphorylation precedes ubiquitylation and proteasomal degradation of Tim in *Drosophila* [8], we next investigated whether SFK-mediated phosphorylation influenced Tim ubiquitylation. We first analyzed the mouse Tim sequence for potential ubiquitylation sites using UbPred [34], a random forest predictor of potential ubiquitylation sites. Four potential ubiquitylation sites were predicted with high confidence (lysine residues 956, 1158, 1159, and 1188), all of which localize to the C-terminal region of the protein. As ubiquitylated *Drosophila* Tim is subject to proteosomal degradation [8], the mTim sequence was also analyzed by Epestfind. This algorithm identifies possible PEST motifs, polypeptide sequences enriched for proline, glutamic acid, serine, and threonine often associated with rapidly degraded proteins [35]. Epestfind identified four putative PEST cleavage sites, also within the mTim C-terminal region (starting at position 652, 972, 1104, 1137). These predictions suggest that mTim may also be regulated by ubiquitylation and proteasomal degradation as reported for its *Drosophila* counterpart [8]. Tim was then co-expressed with each of the SFKs, and the ubiquitylated protein fraction was immunoprecipitated and probed for the presence of Tim via the V5 epitope tag. As shown in Figure 2C, co-expression with each of the SFKs resulted in Tim ubiquitylation. In contrast, no ubiquitylation was observed when Tim was expressed alone. Control blots show consistent expression of both Tim and the active SFKs. These results strongly implicate SFKs in a pathway leading to Tim ubiquitylation.

3.3. SFKs have opposing roles in regulating Tim protein levels

Drosophila Tim degradation is blocked by proteasomal inhibitors as well as ubiquitin depletion, indicating a key role for the 26S proteasome in Tim turnover [8]. Degradation of fly Tim is preceded by tyrosine phosphorylation [8], suggesting that this phosphorylation

event may trigger proteosomal targeting. Therefore we next explored the connection between SFK-mediated phosphorylation and Tim degradation. Tim was expressed in 293T cells either alone or in the presence of the SFKs Fyn, Hck, c-Src and c-Yes as before, followed by determination of relative Tim levels by immunoblotting (Figure 3). Surprisingly, two distinct outcomes were observed. Co-expression with Fyn or Hck resulted in lower levels of Tim, whereas co-expression with c-Src or c-Yes enhanced Tim levels, suggesting a protective role. These data suggest that individual SFKs have non-redundant, opposing influences on Tim protein stability.

To determine whether the opposing roles of SFK co-expression were dependent on kinase activity, Tim was co-expressed with wild-type and kinase-dead variants of Hck and c-Src, the two Src-family members that phosphorylated Tim most strongly. As shown in Figure 4, co-expression of Tim with c-Src led to an increase in Tim protein levels as shown before. However, co-expression with the kinase-dead mutant of c-Src completely reversed this effect, indicating that the observed enhancement of Tim protein levels requires kinase activity. In contrast to c-Src, co-expression with either wild-type or kinase-defective Hck resulted in diminished Tim protein levels, suggesting that interaction with Hck may be sufficient to induce Tim degradation.

3.4. SFK-mediated K63 ubiquitylation determines Tim fate

In general, ubiquitylation is associated with proteosomal degradation. However, growing evidence shows that the type of polyubiquitin chain can have a dramatic impact on the fate of the target protein. Ubiquitin has seven lysines that can be used for protein polyubiquitylation, with linkage through Lys48 (K48-UB) recognized by the proteasome. However, polyubiquitin chains joined through Lys63 (K63-UB) have varied roles in DNA repair processes and signal transduction [36–43]. For example, K63-polyubiquitin promotes the assembly of the multi-protein repair complex at double stranded breaks, thus maintaining genomic stability [44]. K63-ubiquitylation of nerve growth factor receptor tyrosine kinase A (Trk A) is required for internalization and signaling [45]. K63-UB polyubiquitin chains on proteins found in Lewy bodies associated with neurodegenerative diseases appear to promote both the formation of these inclusions and their autophagic clearance [46]. In this case, K63-UB modification may represent a route by which aggregated proteins are diverted away from an overloaded proteasomal machinery [41, 47].

In order to determine whether K63-UB plays a role in SFK-mediated Tim protein accumulation, Tim was co-expressed with each SFK in the presence of a modified form of ubiquitin that can only form polyubiquitin chains through Lys63. Tim was then immunoprecipitated, followed by immunoblotting with an antibody specific for the K63-UB modification. As shown in Figure 5, co-expression of Tim with c-Src or c-Yes led to selective incorporation of K63-UB, while Hck and Fyn did not. This result shows that differential ubiquitin modification on Lys63 may account for the protective effect of these two SFK on Tim expression levels.

Stabilization of Tim resulting from co-expression of c-Src or c-Yes correlated with the K63-UB modification. To determine if modification at K63 was required for this effect, we next co-expressed Tim with these SFKs in the presence of a ubiquitin variant that can only form Lys48 linkages (K48-UB) [48]. Polyubiquitin chains formed via this site are associated with proteosomal degradation. As shown in Figure 6, co-expression of Tim with K48-UB completely reversed the enhancement of Tim levels by both c-Src and c-Yes, supporting the idea that protection of Tim from degradation requires via Lys63-mediated polyubiquitin linkages.

3.5. SFK signals for Tim degradation are dominant to those for protection

Most cell types express multiple members of the Src-kinase family, leading to the question of which SFK-dependent signal influencing Tim protein stability dominates when more than one is present. To address this issue, Tim was co-expressed with SFKs in a pairwise fashion, and Tim levels were assessed by immunoblotting as before. As shown in Figure 7, the enhancement of Tim protein levels observed upon co-expression with c-Src or c-Yes was completely abolished in the presence of either Hck or Fyn. Interestingly, Hck-induced degradation was dominant to the protective influence of c-Src, even if c-Src was transfected first (data not shown).

4. Discussion

In *Drosophila*, Tim is tightly controlled at the protein level via ubiquitylation and proteosomal degradation. This tight control is essential in the function of the circadian autofeedback loop. While the initiation of the degradation cascade is preceded by tyrosine phosphorylation, the identity of the kinase responsible for Tim degradation has not been reported [1–8]. In mammals, Tim levels also appear to be tightly controlled, both in the circadian cycle where Tim protein/RNA levels are cyclically expressed with respect to light [1, 12]. During mammalian embryonic development, Tim is rapidly degraded once cavity formation has concluded [17] and Tim transcript levels decrease with liver and lung organogenesis [18, 49]. Aberrant expression of Timeless is associated with increased breast and colon cancer risk [15, 16], supporting a critical role for cellular mechanisms that control Tim expression. Here we provide new evidence that members of the Src kinase family regulate mammalian Tim protein stability, working in concert to attenuate Tim levels. Tim is an SH3 domain-binding protein for several members of the Src kinase family, and is phosphorylated by SFKs as well. In the case of Hck and Fyn, co-expression led to enhanced Tim turnover, while c-Src and c-Yes promoted Tim stability.

While all four of the Src-family members examined in our study associated with Tim and enhanced its ubiquitylation, co-expression with c-Src and c-Yes unexpectedly enhanced Tim protein levels. The consequences of ubiquitylation are often dependent on the type of linkage used. K48-linked ubiquitin chains are the most common, and constitute a signal for protein degradation by the 26S proteasome [48]. In contrast, K63-linked chains function in a variety of cellular processes, including DNA repair [42], stress responses [37], signal transduction [43], and intracellular trafficking [39–41]. K63-UB is known to modify several proteins that associate with stalled replication forks and maintain genomic stability in response to DNA damage [43, 44]. As Tim is a replication fork-associated factor [32], the K63-UB modification may be involved in localizing Tim for this function in S-phase checkpoint control and in a Src kinase-dependent manner.

Src-family kinase SH3 domains play critical roles in the regulation of kinase activity as well as interaction with partner proteins involved in downstream signaling. For example, Hck and other SFKs recruit the transcription factor STAT3 in an SH3-dependent manner, resulting in transient kinase activation and STAT3 phosphorylation [24]. The HIV-1 accessory protein Nef also binds and activates Hck, Lyn and c-Src through an SH3-mediated interaction, resulting in constitutive kinase activation important for HIV replication [23]. Data presented here show that recognition of Tim by SFKs also involves their SH3 domains. Of the four SFKs studied, the c-Src, Hck and Yes SH3 domains readily bound to Tim, while the Fyn SH3 bound Tim to a lesser degree. No association was observed with the corresponding SFK SH3 domain mutants in which the conserved tryptophan residue on the SH3 domain binding surface was replaced with alanine, suggesting that SFK SH3 domain recognition of Tim occurs through a polyproline type II helix typical of SH3 ligands. Computational analysis of the mouse Tim amino acid sequence with Scansite 2.0 [50] identified two

potential SFK SH3 binding motifs within the C-terminal Timeless-C region, one of which overlaps with a putative PEST sequence. These observations suggest a model in which SH3-mediated recruitment of Tim induces transient SFK activation followed by Tim phosphorylation and release.

Co-expression experiments show that the 'degradative' Hck and Fyn signals are dominant to the 'protective' effects of c-Src and c-Yes on Tim protein levels. This observation suggests that the equilibrium of the SFK-Tim system is tipped towards keeping Tim levels low. The biological relevance of this observation may apply to the formation of embryoid bodies from ES cells, an important cell culture model for pre-implantation embryogenesis. Previous work from our group has shown that Tim drives controlled apoptosis required for cavity formation as ES cells differentiate to embryoid bodies [17]. Tim protein levels rise during cavitation and then decline once the cavity is complete. Interestingly, Hck transcription is rapidly silenced during the onset of embryoid body formation, while c-Src expression and activity remain constant throughout this process [22]. These observations suggest a mechanism in which Hck-mediated regulation of Tim turnover may dominate during the growth and self-renewal of ES cells, but gives way to Src-mediated protection of Tim via K63-linked ubiquitylation as EBs develop [17]. In this way, temporal changes in the expression patterns of individual SFKs may regulate Tim protein levels and downstream signaling related to developmental apoptosis.

We also investigated a role for the proteasome in SFK-enhanced Tim turnover using MG132, a well-known inhibitor of proteasome function. Transfected cells expressing Tim either alone or in the presence of Fyn, Hck, c-Src, or c-Yes were treated in the presence or absence of 25 μM MG132 for 16 h, followed by quantitative immunoblotting for Tim protein in cell lysates. Surprisingly, MG132 treatment did not produce a significant change in Tim protein levels (n = 3; data not shown), suggesting that Tim turnover is not mediated by the proteosome. This raises the intriguing possibility that Tim is degraded by autophagy or another alternative pathway. K48-linked ubiquitin is typically thought of as a signal for proteolytic degradation [51, 52], while K63-linked chains have been linked to autophagy [46, 53, 54]. However, ubiquitylated proteins linked through both K63 and K48 chains accumulate in the brains of mice defective for autophagy, supporting a role for autophagy in the global regulation of ubiquitinated protein levels [55].

One unresolved question involves the identity of the E3 ligase responsible for Tim ubiquitylation in mammalian cells. In *Drosophila*, Tim associates with Jetlag, a putative member of the Skp1/Cullin/F-box SCF E3 ubiquitin ligase complex, in association with light-sensing cryptochromes [9]. Mammalian Tim may also associate with a multiprotein degradation complex involving Src-family kinases and an E3 ligase. Along these lines, we tested c-Cbl as a candidate E3 as it has been shown to interact with c-Src, leading to ubiquitylation of both proteins [56]. We were able to demonstrate c-Cbl interaction with both Tim and SFKs in co-immunoprecipitation assays, suggesting that they may be degraded in a complex similar to *Drosophila* Tim (data not shown). Additional experiments showed that Tim, c-Cbl, and SFKs were degraded as a complex, although the results were inconsistent. Previous studies have shown that c-Src and c-Cbl promote the degradation of each other [57, 58], which may help to explain this experimental variability. Additional studies will be required to implicate c-Cbl in the SFK-mediated turnover of Tim and it biological significance.

5. Conclusion

In summary, the clock protein Tim is an SH3-binding protein and substrate for several members of the Src kinase family. Surprisingly, phosphorylation of Tim by individual Src-

family members led to opposing effects on Tim protein levels. Phosphorylation by Hck and Fyn induced Tim ubiquitylation on K48 and subsequent degradation, while c-Src and c-Yes led to K63-linked ubiquitylation and Tim accumulation within cells. These observations offer a possible mechanism for the differential control of Tim levels in several biological contexts, including early embryonic differentiation where Tim regulates apoptosis.

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Highlights

- The clock protein Timeless is essential for circadian cycle regulation in *Drosophila*
- Timeless also governs cell cycle control and embryonic development in mammals
- Phosphorylation of Timeless by Src-family kinases induces ubiquitylation
- Individual Src-family kinases have opposing actions on Timeless protein turnover
- Stabilization of Timeless by c-Src results from K63-linked ubiquitylation
- Src family members may work in concert to coordinate Timeless function

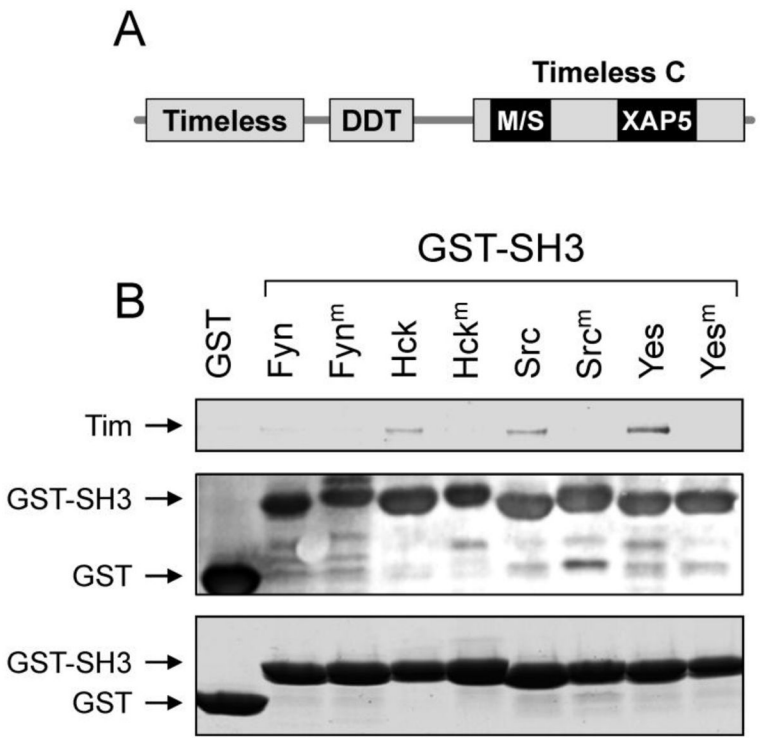

Figure 1. Tim protein organization and SFK-SH3 interaction

A) Tim consists of two large conserved regions, Timeless (amino acids 22–293) and Timeless C (723–1198), as well as the three putative DNA binding motifs DDT (342–376), M/S (Myb/SANT-like; 814–866) and the zinc finger, XAP5 (1084–1144). Drawing not to scale. B) Lysates from 293T cells expressing a V5-tagged version of mouse Tim were incubated with immobilized GST, GST-SH3 fusion proteins for the SFKs shown, or the corresponding binding-defective GST-SH3 mutants (*m*). Following washing, bound proteins were separated by SDS-PAGE, transferred to PVDF membranes and associated Tim was visualized with an anti-V5 antibody. Equivalent capture of GST and GST-SH3 proteins by the glutathione-agarose beads was confirmed by immunoblotting with a GST antibody (middle panel). An image of a Coomassie-stained gel of GST and the GST-SH3 fusion proteins is shown in the bottom panel.

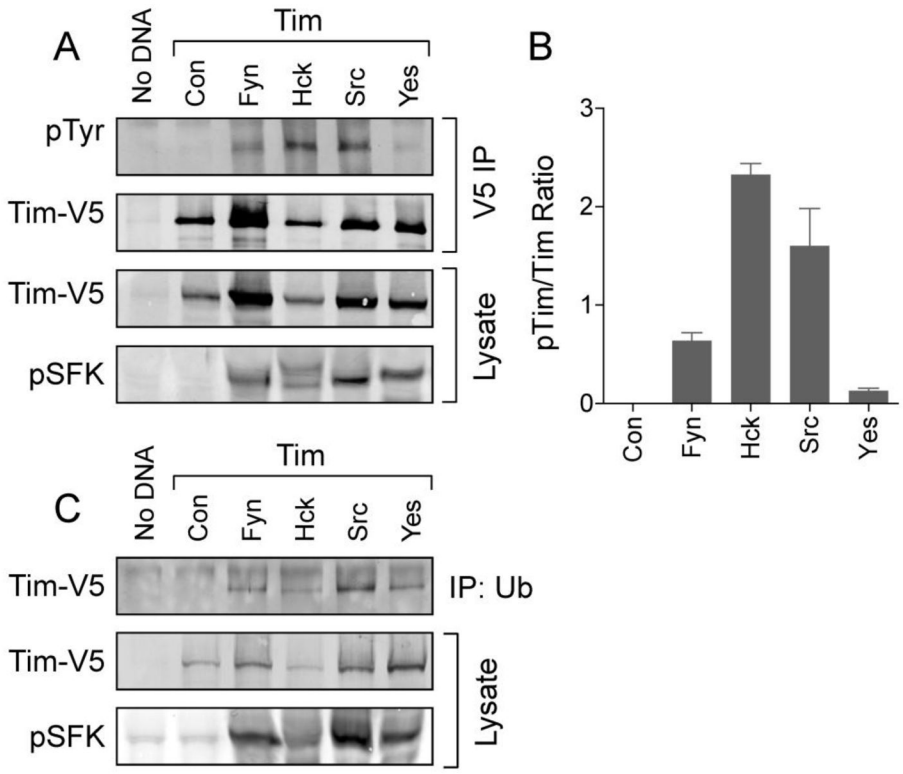

Figure 2. SFK co-expression results in phosphorylation and ubiquitylation of Tim

A) Mouse Tim bearing a C-terminal V5 epitope tag was expressed either alone (Con) or together with the SFKs Fyn, Hck, c-Src, or c-Yes as indicated. Cells transfected with the parent expression vector are also included as a negative control (Vector). Tim was immunoprecipitated from the transfected cell lysates with a V5 antibody and immunoblotted for Tim protein recovery (Tim-V5) and tyrosine phosphorylation (pTyr). Active SFKs (pSFK) and Tim protein expression were confirmed in the transfected cell lysates, with actin as a loading control. B) Tim phosphotyrosine content was normalized to the amount of Tim protein captured in each IP, and quantified using Image J [27]. The bargraph shows the average ratio ± S.E.M. (n=3). C) Tim-V5 and SFKs were co-expressed in 293T cells followed by immunoprecipitation with an anti- Ubiquitin antibody. Tim-V5 was visualized in the ubiquitin immunoprecipitates by immunoblotting for the V5 epitope. Tim and active SFK expression were validated in lysate blots as per part A.

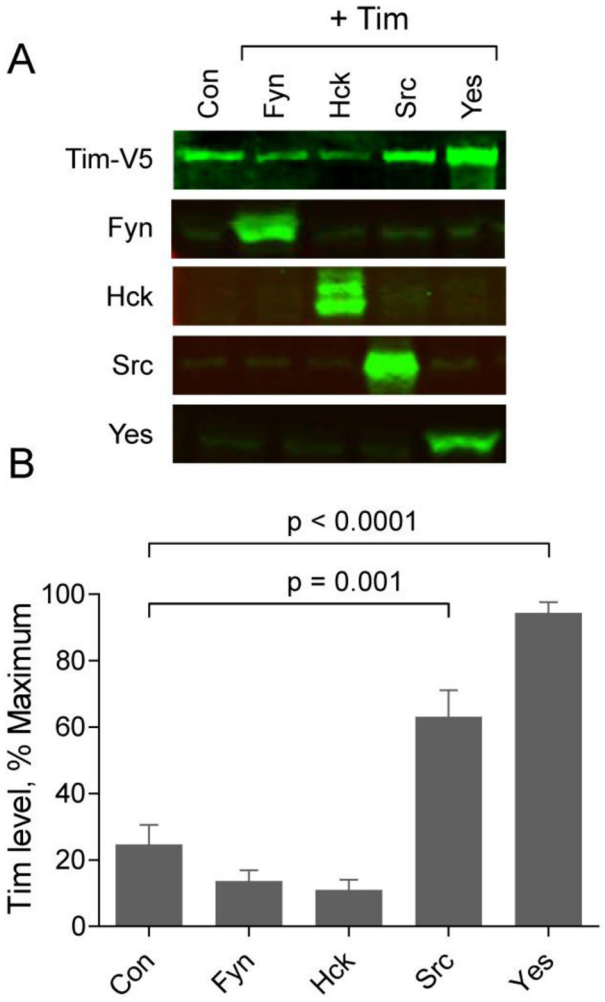

Figure 3. Differential effects of individual Src-family members on Tim protein levels

A) Tim-V5 was expressed either alone (Con) or together with the SFKs shown in 293T cells. Tim-V5, SFK, and Actin protein levels were quantitated by immunoblotting using the Odyssey infrared imaging system. A representative set of blots is shown. B) Tim levels were first normalized to Actin, and then plotted as a ratio of Tim alone to Tim co-expressed with the SFKs. The bargraph shows the average ratio ± S.E.M. (n=3). Statistical significance was established using an unpaired Student's t-test.

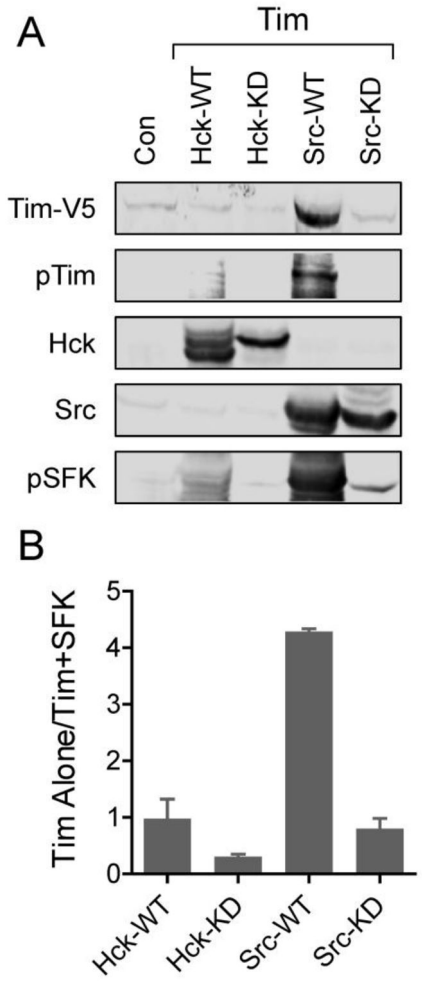

Figure 4. Protection of Tim degradation by c-Src is kinase-dependent

A) Tim-V5 was expressed either alone (Con) or together with either the wild-type (WT) or kinase-dead (KD) versions of Hck and Src in 293T cells as indicated. Tim-V5, phosphorylated Tim (pTim), Hck, c-Src and active SFK (pSFK) levels were quantitated by immunoblotting using the Odyssey infrared imaging system. A representative set of blots is shown. B) Tim levels are plotted as a ratio of Tim alone to Tim co-expressed with the SFKs. The bargraph shows the average ratio ± S.E.M. (n=3).

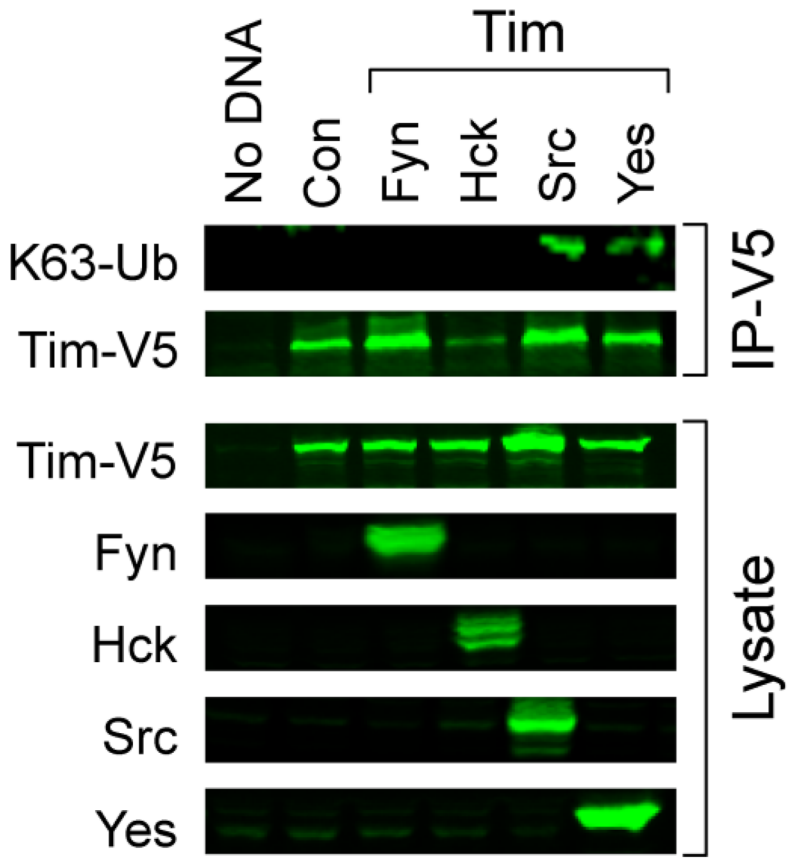

Figure 5. c-Src and c-Yes, but not Hck or Fyn, induce ubiquitylation of Tim on K63

A) Tim-V5 was expressed either alone (Con) or together with the SFKs Fyn, Hck, c-Src, or c-Yes as indicated. Cells transfected with the parent expression vector are also included as a negative control (Vector). Tim was immunoprecipitated from the transfected cell lysates with a V5 antibody and immunoblotted for Tim protein recovery (Tim-V5) and for K63-selective ubiquitylation (K63-Ub). Expression of Tim-V5 and individual SFK proteins was confirmed in the transfected cell lysates. This experiment was repeated three times with comparable results; a representative example is shown.

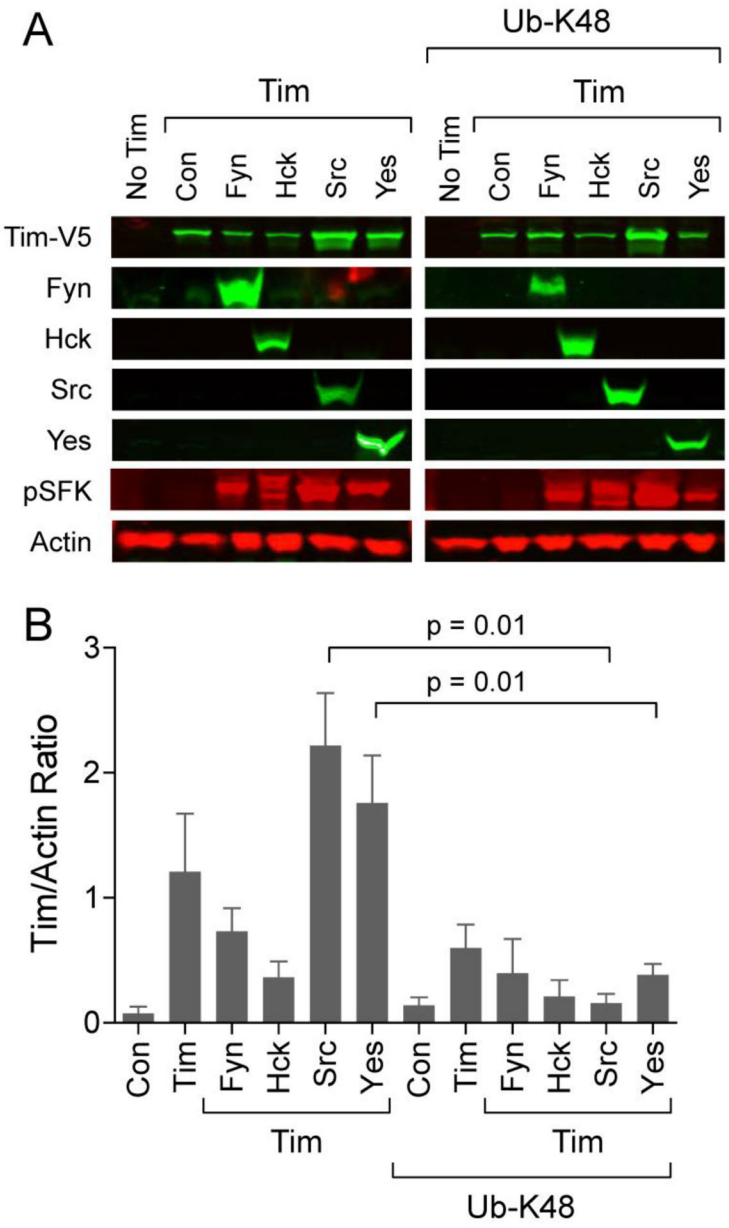

Figure 6. K48 ubiquitin enhances the degradation of Tim

A) Tim-V5 was expressed either alone (Con) or together with the SFKs Fyn, Hck, c-Src, or c-Yes as indicated. Cells transfected with the parent expression vector are also included as a negative control (Vector). A parallel set of cultures was also transfected with an expression plasmid for Ubiquitin-K48 (right panels). Tim-V5, SFKs, active SFK (pSFK) and Actin levels were quantitated by immunoblotting using the Odyssey infrared imaging system. A representative set of blots is shown. B) The bargraph shows the average Tim to Actin ratios ± S.E.M. (n=3). Statistical significance was established using an unpaired Student's t-test.

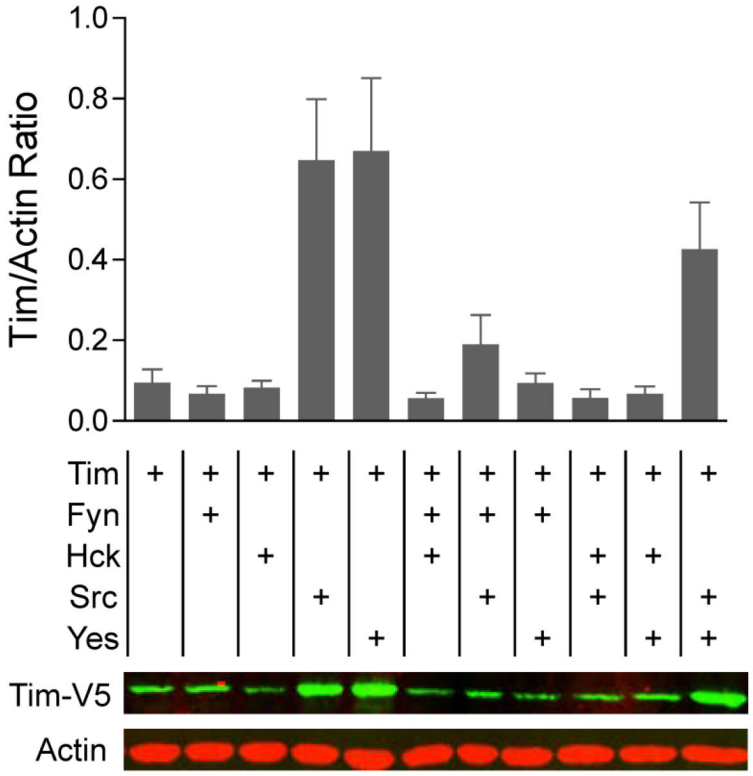

Figure 7. SFK signals for Tim degradation are dominant

Tim-V5 was expressed alone, together with the SFKs Fyn, Hck, c-Src, or c-Yes, or with the combinations of SFKs indicated. Tim-V5 and Actin levels in the transfected cell lysates were quantitated by immunoblotting using the Odyssey infrared imaging system. A representative set of blots is shown at the bottom. The bargraph shows the average Tim to Actin ratios ± S.E.M. (n=6). Unpaired Student's t-tests confirmed statistically significant differences in Tim levels when co-expressed with c-Src ± Fyn ($p = 0.02$), c-Src ± Hck ($p = .003$), c-Yes ± Fyn ($p = .01$), and c-Yes ± Hck ($p = .008$).