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***The E3 ligase TTC3 facilitates ubiquitination and degradation of phosphorylated Akt***

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### ***Abstract***

The serine threonine kinase Akt is a core survival factor that underlies a variety of human diseases. Although regulatory phosphorylation and dephosphorylation have been well documented, the other post-translational mechanisms that modulate Akt activity remain unclear. We show herein that TTC3 (tetratricopeptide repeat domain 3) is an E3 ligase that interacts with Akt. TTC3 contains a canonical RING-finger motif, a pair of TPR (tetratricopeptide) motifs, a putative Akt phosphorylation site, and nuclear localization signals, and is encoded by a gene within the Down Syndrome (DS) Critical Region on chromosome 21. TTC3 is an Akt-specific E3 ligase that binds to phosphorylated Akt and facilitates its ubiquitination and degradation within the nucleus. Moreover, DS cells exhibit elevated TTC3 expression, reduced phosphorylated Akt, and accumulation in the G<sub>2</sub>M phase, which can be reversed by TTC3 siRNA or Myr-Akt. Thus, interaction between TTC3 and Akt may contribute to the clinical symptoms of DS.

## ***Introduction***

Serine threonine kinase Akt regulates diverse cellular processes, including cellular survival, proliferation, cell cycles, cytoskeletal organization, vesicle trafficking, glucose transport, and platelet function. Deregulation and altered Akt activity underlies a variety of human diseases, including cancers, glucose intolerance, schizophrenia, viral infections, and autoimmune diseases. Therefore, the PI3K-Akt network provides an ideal target for drug development.

Akt activation is regulated primarily by phosphorylation at two critical sites: threonine 308/309/305 and serine 473/474/472 (in Akt1, Akt2, Akt3, respectively). Phosphorylation of both residues is required for maximum kinase activity. PDK1 (3-phosphoinositide-dependent protein kinase 1) has been identified as a primary kinase that will phosphorylate Akt at Thr 308. Kinases that activate Ser 473 are known as “PDK2”, that include mitogen-activated protein (MAP), kinase-activated protein kinase-2 (MK2), integrin-linked kinase (ILK), p38 MAP kinase, protein kinase C $\alpha$  (PKC $\alpha$ ), Protein kinase C $\beta$ II, rictor-mTOR complex (mTORC2), double-stranded DNA-dependent protein kinase (DNK-PK), autophosphorylation, and ataxia telangiectasia mutated (ATM) gene product (Brazil et al., 2004; Manning and Cantley, 2007). Akt is also known to interact with various intracellular molecules that modulate Akt activation and its downstream signals (Du and Tschlis, 2005; Noguchi et al., 2007). Recent work has demonstrated that protein phosphatases, such as PH domain leucine-rich repeat protein phosphatase (PHLPP), can silence Akt by specifically dephosphorylating the hydrophobic motif of Akt (reviewed in

Manning and Cantley, 2007). The phosphorylation-dephosphorylation mechanisms are thought to primarily control the PI3K-Akt signaling, however, the involvement of ubiquitination-proteasomal pathways associated with Akt signaling have been reported (Dickey et al., 2008; Facchinetti et al., 2008; Xiang et al., 2008).

Ubiquitin, a 76-residue protein, is covalently associated with protein substrates. Protein ubiquitination is mediated through three enzyme families (E1, E2, and E3). Ubiquitin is activated first by a ubiquitin-activating enzyme (E1) through an ATP-dependent reaction to form an E1-thioester linkage, and the activated ubiquitin is transferred to a member of the ubiquitin-conjugating enzyme E2 family. Ubiquitin-protein ligase (E3) mediates the transfer of ubiquitin from E2 to the substrate protein by promoting the formation of an isopeptide bond between the ubiquitin (Ub) carboxyl-terminus and specific lysine side chains on the substrate (Hochstrasser, 2000; Pickart and Eddins, 2004).

In addition to the cellular waste disposal machinery via the 26S proteasome, ubiquitination can regulate wide varieties of cellular functions, including cell-surface-receptor turnover, gene transcription, cell cycle transition, apoptosis, proliferation, DNA repair, checkpoints, and differentiation (Pickart and Eddins, 2004). Thus, the activity of the ubiquitin system is dependent on the specificity of the E3 ubiquitin ligases (Verma et al., 2004). To date a direct connection between the Akt and ubiquitin pathways has not been clear as an Akt-specific E3 ligase which can bind to Akt and induce ubiquitination of Akt has not been identified.

By using yeast two-hybrid systems with full length human Akt2 as a bait, we found a stable interaction between the TTC3 and Akt. TTC3, which harbors a RING finger motif for

ubiquitin-protein ligase and putative Akt phosphorylation motifs is located at chromosome 21 within the Down Syndrome Critical Region (DSCR) (Antonarakis et al., 2004; Rachidi et al., 2000; Reeves et al., 2001; Tsukahara et al., 1998). Herein, we have demonstrated that TTC3 is a novel Akt-specific E3 ligase that binds phosphorylated Akt and can silence its activity via a proteasomal cascade. This finding could underlie important clinical manifestations of Down syndrome (DS), the most common genetic disorder of humans.

## ***Results***

### ***TTC3 (Tetratricopeptide repeat domain 3) is a binding partner for Akt***

Using human Akt2 as a bait in yeast two-hybrid screening, we identified two independent clones encoding a partial cDNAs of human TTC3 (Tetratricopeptide repeat domain 3)[ (AA from 890 to 1100) and (AA from 870 to 1102)] (Tsukahara et al., 1998).

TTC3 originally was identified as one of the candidate genes for DS located within the Down Syndrome Critical Region (DSCR) of human chromosome 21 (Antonarakis et al., 2004; Rachidi et al., 2000; Reeves et al., 2001). The open reading frame for TTC3 is 2025 amino acids in length and has 76% homology between humans and mice. Identified motifs of TTC3 include a pair of TPR motifs (Lamb et al., 1995), nuclear localizing signals, and the canonical C-terminal H2-RING domain with a short motif rich in cysteine and histidine residues (CEICHEVFKSKNVRVLKCGHKYHKGCFKQWL KGQSAC PACQ) (**Fig. 1A**).

Three Akt isoforms (Akt1, Akt2, and Akt3) are present in the human genome, and are greater than 85% homologous at the amino-acid level. Using 293T cells, all three Akt isoforms, but not PDK1 or PrKA, interacted with TTC3 (**Fig. 1B** and **1D**). The interactions between endogenous TTC3 and the three isoforms of Akt (Akt1, Akt2, or Akt3) were further confirmed in co-immunoprecipitation experiments (**Fig. 1C**).

To further define the interacting domains within TTC3 with Akt, a series of TTC3 subfragments were generated in mammalian expression vectors (see Fig. 1A). Transfection of these vectors into 293T cells demonstrated the Int-TTC3 vector (Amino acids from 491-1539 of TTC3) could mediate a stable interaction with Akt during co-immunoprecipitation (**Fig. 1E**).

The Akt protein possesses the N-terminal PH (Pleckstrin Homology) domain and the C-terminal catalytic kinase domain. The interaction between TTC3 and Akt was mediated primarily through the C-terminal domain of Akt in co-immunoprecipitation assays (**Fig. 1F**). Since the initial TTC3: Akt interaction was identified using Akt2, we focused our investigation of the biological significance of this interaction using Akt2, unless specified.

### ***TTC3 is a E3 ubiquitin ligase for Akt***

The TTC3 gene encodes canonical E3 RING-finger motif at the C-terminal end (see Fig. 1A). One of the most remarkable features of the ubiquitin-conjugating pathway is the diversity of substrate specificity, which is determined essentially by the E3 ligases. To determine whether TTC3 actually has an ubiquitin ligase activity, we generated recombinant TTC3 protein (**Fig. S1**) and performed *in vitro* ubiquitination assays. First, we examined the preference of TTC3 among the eight human E2 enzymes in the presence of E1. TTC3 was polyubiquitinated only in the presence of Ubc4 or Ubc5C as E2 enzymes (**Fig. 2A**). Next, to examine the requirement of TTC3 for ATP, ubiquitin, E2 or TTC3 by systemically deleting one component at a time from the *in vitro* ubiquitination assays reaction mixture. Immunoblot analysis using an anti-ubiquitin antibody revealed that TTC3 exhibits ubiquitination activity only in the presence of E1, E2 (Ubc5C), ubiquitin, ATP and TTC3 (**Fig. 2B**). Furthermore, TTC3 preferentially polyubiquitinated phosphorylated Akt compared with unphosphorylated Akt (**Fig. 2C**, also see Fig. 3A-D), supporting the notion that TTC3 is acting as an E3 ligase for Akt.

Since TTC3 interacts specifically with Akt, we asked whether TTC3-dependent

ubiquitination is specific for Akt. Using 293T cells transfected with wild type TTC3 and HA-tagged human Akt, we observed efficient poly-ubiquitinated Akt (**Fig. 2D**, left panel). In contrast, TTC3 expression did not result in ubiquitination of, but not AK1 kinase (endogenous) or citron kinase (**Fig. 2D**, right panel and data not shown). The TTC3 RING-finger motif is required for Akt ubiquitination, since wild-type TTC3 (but not  $\Delta$ RF-TTC3, which lacks the RING-finger motif) induced ubiquitination (**Fig. 2E**). Moreover, the binding-defective mutant ( $\Delta$ AB) or N-term TTC3 exhibited significant low levels of ubiquitination activity (**Fig. S2**), which correlated well with decreased Akt degradation (**Fig. S3**). These results suggest that interaction of the TTC3-Akt is important for biological function.

K48R-Ubiquitin, in which lysine 48 of ubiquitin is mutated into arginine, completely abolished efficient ubiquitination demonstrating that TTC3 conjugated with K48-linked polyubiquitin chain(s) onto Akt (**Fig 2F**). Knock down of TTC3 by siRNA failed to induce Akt ubiquitination (**Fig. 2G**). Furthermore, TTC3 induced ubiquitination more efficiently on wild type Akt compared with T308A-S473A-Akt in an in vitro ubiquitination assay (**Fig. 2H**).

### ***TTC3 preferentially binds to phosphorylated Akt, and facilitates its proteasomal degradation***

Phosphorylation plays a key role in ubiquitination by E3 enzymes and subsequent proteolysis (Gao and Karin, 2005; Hunter, 2007). Therefore, we next verified the phosphorylation-dependent interactions of TTC3 with Akt. The interaction between

endogenous Akt-TTC3 was augmented when Akt was activated/phosphorylated (**Fig. 3A**). This finding correlated with efficient ubiquitination of Akt in both in cellular (**Fig. 3C**) and *in vitro* ubiquitination assays (see **Fig. 2C**). Furthermore, TTC3 bound to the phosphorylated Akt more efficiently compared with non-phosphorylated Akt in a GST-pull down assay (**Fig. 3B**) and in co-immunoprecipitation assays (**Fig. 3D**).

Increasing amounts of TTC3-siRNA, but not control siRNA, reduced the TTC3 expression in a dose-dependent manner (**Fig. 3E**). TTC3 expressions were correlated inversely with phosphorylated Akt (Thr308 and Ser473) and phosphorylated FKHR without affecting MAP kinases. In these experiments, total Akt showed no increase with the addition of TTC3-siRNA, since only a small fraction of Akt is phosphorylated. Furthermore, TTC3 induced Akt degradation via a proteasomal cascade when Akt is activated by Myr-Akt (**Fig. 3F**).

#### ***Phosphorylation of TTC3 at Ser 378 is required for efficient biological function***

Using “Scan Site” (Obenauer et al., 2003), Ser 378 of TTC3 (TPRSLSAP) was identified as a putative phosphorylation site for Akt. Immunoprecipitation of Flag tagged TTC3 followed by immunoblotting and detection with an anti-phospho Akt antibody indicated that Akt activation induced phosphorylation of wild-type TTC3 (top panel, lane 5), but failed to induce phosphorylation of S378A-TTC3 in Myr-Akt transfected cells by anti-phospho Akt-substrate-specific immunoblot after Flag (TTC3) immunoprecipitation (**Fig. 4A**). Analogous to this finding,  $\Delta$ N-TTC3, which also lacks S378 of TTC3, failed to be phosphorylated by Akt (data not shown). In an *in vitro* Akt kinase assays,

phosphorylation of wild type TTC3, but not S378A-TTC3 (**Fig. 4B**) was detected. We then performed an Akt-siRNA experiment to suppress the endogenous expression of Akt and verify the levels of phosphorylation of TTC3. The levels of TTC3 phosphorylation detected by anti-Akt substrate antibody (Cell Signaling # 9611) were diminished its intensity (**Fig. 4C**). Together, these observations support that TTC3 is a phosphorylation target of Akt both in an *in vitro* and in a cellular context, although we cannot rule out the possibility that other kinases such as S6K might be able to phosphorylate TTC3 S378 *in vivo*.

Functionally speaking, Ser378 appears to be important for the regulation of TTC3's biological activity. Cells transfected with S378A-TTC3 or  $\Delta$ RF-TTC3 demonstrated lower ubiquitination action compared with wild-type TTC3 (**Fig. 4D**). In addition, wild-type TTC3, but not S378A-TTC3, could induce Akt degradation in a time-dependent manner (**Fig. 4E**). Wild-type TTC3 could also induce mitochondrial membrane depolarization more efficiently compared with S378A-TTC3 when measured by rhodamine 123 (Rho123) transport (**Fig. 4F**).

Akt is known to activate IKK, resulting in transactivation of NF $\kappa$ B as one its anti-apoptotic responses. In a luciferase reporter assay, wild-type TTC3 inhibited NF $\kappa$ B transactivation more efficiently compared with S378A-TTC3(**Fig. 4G**). Furthermore, proliferation assays also indicated that wild-type TTC3 had a higher level of anti-proliferation activity compared with S378A-TTC3 (**Fig. 4H** and **Fig 6A**). However, addition of Myr-Akt could block this effect of TTC3 (**Fig. S4A-C**). Together, these results establish that Serine 378 of TTC3 plays an important role in Akt-dependent activities

***TTC3 associates with Akt and induces polyubiquitination and proteolytic degradation of Akt within the nucleus***

TTC3 contains a pair of “nuclear localization” signals (see Fig. 1A). Activated Akt is known to translocate to the nucleus (Andjelkovic et al., 1997). Therefore, we next compared the levels and the distribution of ubiquitination and phosphorylation of Akt between the cytoplasmic (C) and nuclear fraction (N). TTC3 facilitated efficient polyubiquitination of endogenous Akt (**Fig. 5A**), and transfected Akt (**Fig. S5**), within the nucleus. Consistently, nuclear Akt, which is phosphorylated, is degraded more efficiently compared with the cytoplasmic Akt by TTC3 via a proteasomal cascade (**Fig. 5B** and **5C**).

Confocal microscopy was utilized to further define the nuclear co-localization of Akt with TTC3 by using a rabbit anti-TTC3 antibody (**Fig. S6A** and **S6B**). During interphase, TTC3 was observed to co-localize with Akt primarily within the nucleus in both NIH 3T3 cells and Hs52.Sk cells (DS skin fibroblast, ATCC), in which TTC3 was endogenously expressed (**Fig. 5D**, **5E**, and **5G**). Furthermore, TTC3 preferentially co-localized with phosphorylated Akt in the nucleus, and concentrated around the kinetochore during the mitosis (**Fig. 5E** and **5F**). Addition of MG132, a proteasomal inhibitor, enhanced the co-localization of Ser473 phosphorylated Akt and Myr-Akt with endogenous TTC3 (**Fig. S7** and **S8**). These results demonstrated that TTC3 preferentially co-localized with Akt in the nucleus throughout the cell cycle.

***Functional interaction of Akt-TTC3 in DS cells***

Ubiquitination can regulate a wide variety of cellular functions (Pickart and Eddins,

2004).

Introduction of wild-type TTC3 significantly inhibited cell proliferation compared with S378A-TTC3 or control transfected cells (**Fig. 6A**, also see **Fig. 4H**). In contrast, suppression of TTC3 by siRNA augmented cellular proliferation in HT1080 and MCF7 cells (**Fig. 6B** and data not shown). Moreover, ectopic re-introduction of wild type TTC3 clearly reverted or inhibited the augmented proliferation triggered by the four different TTC3-siRNAs (**Fig. S9**).

The gene for TTC3 is located within the DSCR(Antonarakis et al., 2004), and is reported to be modestly overexpressed in the tissues from the DS individuals (Baldus et al., 2004). Ts65Dn, a mouse model for DS exhibits a 19% elevation of the TTC3 transcript and has a demonstrated learning deficiency (Antonarakis et al., 2004; Saran et al., 2003).

Both Hs52.Sk cells and CMK85 cells (Sato et al., 1989), which are derived from DS individuals, showed increased TTC3 expression with undetectable levels of phosphorylated Akt (**Fig. 6C**). In Hs52.Sk cells, TTC3 co-localized with Akt predominantly within the nucleus (see Fig. 5G). Introduction of TTC3-siRNA into CMK85 cells exhibited reduced TTC3 expression, which was correlated inversely with phosphorylated Akt expression (**Fig. 6D**).

Consistent with the previous report that Akt activation is known to overcome the G<sub>2</sub>M check-point (Kandel et al., 2002), transfection of TTC3, which presumably facilitates proteasomal degradation of Akt, exhibited an accumulation of cells at the G<sub>2</sub>M check-point, and a 70% decrease in the G<sub>1</sub>/G<sub>2</sub>M (**Fig. 6E**). Similarly, introduction of TTC3 into HDF cells (**Fig. 6F**) or Jurkat cells (**Fig. 6G**) also reduced the exhibited G<sub>1</sub>/G<sub>2</sub>M ratio by

consistent with 293T cells (see Fig. 6E). Analogous to this finding, both Hs52.Sk cells (**Fig. 6F**, bottom panel) and CMK85 cells (**Fig. 6G**, bottom panel), with elevated levels of TTC3 expression, exhibited a significant accumulation of G<sub>2</sub>M cells as detected by a relative decrease in the G<sub>1</sub>/G<sub>2</sub>M ratio.

Introduction of TTC3-siRNA into CMK85 DS cells, which enhanced the expression of phosphorylated Akt (see Fig. 6D), exhibited 450% increase in the G<sub>1</sub>/G<sub>2</sub>M ratio (**Fig. 6H**). In contrast, CMK85 cells transfected with Myr-Akt overcame the G<sub>2</sub>M phase, hence exhibiting 300% increase of the G<sub>1</sub>/G<sub>2</sub>M ratio (**Fig. 6I**).

Together with the demonstration that TTC3 can enhance apoptotic phenotypes (see Fig. 4. F-H), these results further supported the functional significance of TTC3-Akt interaction.

## *Discussion*

By means of a yeast two-hybrid screening, Akt was found to interact specifically with TTC3, which is located within the DSCR on human chromosome 21 (Antonarakis et al., 2004; Rachidi et al., 2000; Reeves et al., 2001; Tsukahara et al., 1998). TTC3 is a 2025 amino acid protein, which is 76% conserved between human and mice and contains, canonical H2-ring-finger domain consisting of a short motif rich in cysteine and histidine residues (Hochstrasser, 2000; Pickart and Eddins, 2004).

By using immunoprecipitation and *in vitro* ubiquitination assays, we confirmed that TTC3 interacted with Akt and functions as a specific E3 ligase for Akt (**Fig. 7**). The interaction between Akt and TTC3 was mediated through the C-terminal kinase domain of Akt. Since the kinase domain of Akt is highly conserved (90-95%) among the three Akt isoforms (Akt1, Akt2, and Akt3), it is logical that TTC3 was able to interact with all three isoforms of Akt. We show that AK1, another serine threonine kinase, could not be ubiquitinated by TTC3. TTC3 also is shown to inhibit neuronal differentiation via RhoA and Citron kinase (Berto et al., 2007). However analogous to AK1 kinase, TTC3 failed to induce ubiquitination of citron kinase nor did it degrade PDK (**Fig. S10**; data not shown).

*In vitro* ubiquitination assays demonstrated that Ubc4 and Ubc5, as E2 enzymes, were required for TTC3-dependent ubiquitination. The observation that either Ubc4 or Ubc5C were required for TTC3-dependent ubiquitination is logical since Ubc4 and Ubc5C share 92% identity at the amino-acid level. The number of ubiquitin-conjugating enzymes (known as E2) present in the human genome is approximately one-tenth that of the E3 ligases. Thus, it is possible that in various cellular environments, different combinations of

E2s and E3s exhibit distinct functions for their specific enzymatic targets.

Akt is known to be regulated primarily by phosphorylation at two sites: a conserved threonine residue in the activation loop (T308) and a serine/threonine residue in a hydrophobic motif (S473) (Alessi et al., 1996 ; Brazil et al., 2004; Manning and Cantley, 2007). Several lines of evidence have supported that phosphorylation plays a key regulatory role in E3 function (Gao and Karin, 2005; Hunter, 2007). To support this notion, TTC3 interacted preferentially with phosphorylated Akt and induces polyubiquitination and subsequent degradation of Akt in the nucleus. Furthermore, PDGF stimulation, which induces Akt activation, resulted in enhanced interaction between Akt and TTC3, an Akt-specific ubiquitin ligase.

Using scan site (<http://scansite.mit.edu/>)(Obenauer et al., 2003; Yaffe et al., 2001), Ser 378 was identified as a putative Akt phosphorylation site. This site was conserved in human, mice, and rats. TTC3 Ser378 does not match the consensus “-5 Arg” within the Akt phosphorylation motif (TPRSLSAP), however, there were several molecules in which “-5 Arg” was not conserved including CREB (LSRRPS) (Kato et al., 2007) , SRPK2 (HDRSRT)(Jang et al., 2009). In both in cellular and *in vitro* assays, we showed that wild type TTC3, but not S378A, could be phosphorylated by Akt. However, we cannot exclude that other kinases such as S6K and other kinases are able to phosphorylate TTC3 *in vivo*.

Functionally, this phosphorylation of TTC3 S378 was required for all the activities we identified for TTC3. In this regard, it is of interest to define structurally how phosphorylated TTC3 and phosphorylated Akt (preferentially on Thr 305/308 of Akt) can be interacted for a specific functional target.

TTC3 also contains a pair of nuclear localizing signals. Consistently, TTC3 bound to Akt in a phosphorylation dependent manner predominantly in the nucleus. Furthermore, by confocal microscopy, we observed that endogenous TTC3 diffusely distributed in the nucleus essentially throughout the cell cycle. However, during the mitotic phase, TTC3 appeared to co-localize with Akt at and around the kinetochore, and the midzone of the cell. This observation seems to be logical since a pair of TPR motifs present in TTC3 is suggested to play a role in the mitotic cell division (Lamb et al., 1995; Rachidi et al., 2000).

Recently, work has demonstrated the connection between ubiquitination-proteasomal pathways and Akt signaling has attracted attention (Basso et al., 2002; Xiang et al., 2008). Specifically the interaction of Hsp90 with Akt was able to affect its stability. CHIP (Chaperon-associated ubiquitin ligase), a E3 ligase family with a TPR motif, was shown to interact with Hsp90 and control the ubiquitination of Akt (Dickey et al., 2008; Ramsey et al., 2000). Phosphorylation of Akt at the Turn motif (TM) is also reported to control the stability of Akt (Facchinetti et al., 2008). Based on co-immunoprecipitation and nuclear ubiquitination-degradation assays, TTC3 appeared to preferentially bind phosphorylated Akt and target it for proteasomal degradation. Since Akt activation enhanced the interaction between TTC3 and Akt, it is possible that Akt TM phosphorylation by mTORC2 will help to stabilize Akt and prevent TTC3-induced proteasomal degradation.

The TTC3 gene is located within the DSCR, the trisomic locus responsible for DS (Antonarakis et al., 2004). Individuals with DS exhibit a wide variety developmental abnormalities including mental retardation, characteristic facial and physical appearances, congenital cardiac malformations, and a high frequency of association with childhood

leukemia (Reeves et al., 2001; Sato et al., 1989; Shinohara et al., 2001).

Although dosage imbalance of the genes located within the DSCR is believed to play a role in the molecular pathogenesis of DS, due to the complexities of the clinical manifestation of DS, the molecular mechanisms remain to be determined (Antonarakis et al., 2004; Reeves et al., 2001). Increased TTC3 expression in the tissues derived from DS individual also has been reported (Baldus et al., 2004; Saran et al., 2003). Non-chimeric polytransgenic 152F7 mice that express DSCR, DSCR5, TTC3, and DYRK1A genes exhibited learning and memory impairment. Another animal model for DS, Ts65Dn, in which trisomic for App-Znf295, also exhibited a 19% elevation of TTC3 transcript (Antonarakis et al., 2004; Reeves et al., 2001; Saran et al., 2003).

In DS-derived cells, we show decreased levels of phosphorylated Akt and relative accumulation of cells at the G<sub>2</sub>M phase of the cell cycle. We demonstrate that TTC3 could enhance an apoptotic phenotype, and inversely, suppression of TTC3 could enhance proliferation. These observations may be explained by a TTC3-Akt interaction, since apoptotic cells were shown to be increased in DS neurons with generation of reactive oxygen species, which is known to be regulated by Akt (Busciglio and Yankner, 1995; reviewed in Manning and Cantley, 2007).

The tissue distribution of TTC3 is located predominantly in neuronal cells (Rachidi et al., 2000; Tsukahara et al., 1998). DS often exhibits subnormal brain development with mental retardation and high incidence of onset of Alzheimer diseases at younger ages. Brain morphology in DS is characterized by a reduced cortical size, a disproportionately small cerebellum, and loss of cholinergic neurons (Reeves et al., 2001; Sato et al., 1989;

Shinohara et al., 2001). In this regard, it is of noteworthy that Akt3-deficient mice exhibited a reduced brain size, affecting all major brain regions consistent with the restricted expression of Akt3 in neurological tissues (Easton et al., 2005; Peng et al., 2003; Tschopp et al., 2005). Although further studies are required, our current work provides a possible clue for the connection between altered interaction between TTC3 and Akt and the development of clinical manifestations of DS, the most common genetic disorder in humans.

## ***Experimental Procedures***

Please refer the detailed experimental procedures in the supplemental information.

### **Yeast Two-Hybrid screening**

Yeast two hybrid assays were essentially performed as described previously (Laine et al., 2000). Y190 cells (Clontech) were transformed by the lithium acetate method with the "bait plasmid" (Akt2/PAS2-1).  $\beta$ -gal positive clones were subsequently mated with Y187 yeast carrying pAS1-CYH2 without an insert or with SNF-1 or p53 to determine the specificity for the interaction.

### **Construction of TTC3 expression vectors**

Plasmid vector containing a partial cDNA fragment of human TTC3 was purchased from imaGenes (RZPD). The 5'-end of the cDNA of human TTC3 was extended by PCR amplification with cDNA from human Peripheral Blood Lymphocyte (PBL). The resulted PCR amplified full length TTC3, was subcloned into the pCMV-Tag2 vector (Stratagene).

### **Cell culture**

[293T cells (ATCC), NIH3T3 cells (ATCC), f3 (normal skin fibroblast), HDF (normal human dermal fibroblasts, Cell Applications, Inc. #106-05a), or Hs52.Sk cells (Down syndrome patient-derived skin fibroblast, ATCC,CRL-7031)] were cultured in DMEM and [Jurkat cells (ATCC) or CMK85 cells [Down syndrome patient-derived megakaryoblastic leukemia cells(Sato et al., 1989)] were cultured in RPMI1640 both in the presence of 10%

FBS and Pen/Strep at 37°C with 5% CO<sub>2</sub> unless specifies.

### **Co-immunoprecipitation experiments**

Methods are essentially described in (Laine et al., 2000) and presented in the supplemental information.

### **Generation and Purification of Recombinant TTC3**

Methods are essentially described in (Laine et al., 2000) and presented in the supplemental information.

### **Ubiquitination assays**

An *in vitro* ubiquitination assay was performed as described (Lorick et al., 1999). Reaction mixtures with recombinant TTC3, were incubated for 2 hr at 30°C, and immunoblotted. For the cellular ubiquitination experiments, cells were transfected with indicated TTC3 constructs or siRNA, treated with MG132, and lysed, immunoprecipitated, and resolved onto SDS-PAGE, and immunoblotted.

### **siRNA**

Cells were transfected with HA-Ubiquitin, treated with TTC3 siRNA (Invitrogen), and immunoprecipitated with Akt and immunoblotted with anti-HA Abs. 293T cells (ATCC) transfected with wild type TTC3 were transfected with TTC3 siRNA or control (IDT) by PEI, harvested, and immunoblotted by indicated antibodies. Hs52.Sk cells were transfected

with Akt-siRNA ( Cell signaling) using CUY21 Pro-vitro (NEPA GENE Co. Ltd).

### **Myr-Akt induced Akt degradation by TTC3**

293T cells were transfected with indicated plasmids with (or without)  $\Delta$ -PH Myr-Akt (Kohn et al., 1996). The cells were treated with 17-Allylamino-17-demethoxygeldanamycin (17AAG), a inhibitor for HSP90 (Basso et al., 2002), for 12 hours before CHX treatment (at time 0) with or without MG132 (Sigma). Cells were harvested at indicated time points, resolved onto SDS-PAGE, and immunoblotted.

### **Brij97 cell lysis buffer:**

The formulation of Brij buffer(Laine et al., 2000) was described in the supplemental information.

### **Akt induces phosphorylation of S378**

293T cells were transfected with indicated plasmids with (or without, as indicated) Myr-Akt (Upstate #21-151). 72 hours later, the cells were harvested, lysed, immunoprecipitated, resolved onto SDS-PAGE, and immunoblotted using ECL.

### **Co-localization experiment using a confocal microscopy**

NIH 3T3 cells or Hs52.Sk cells (ATCC) were cultured in DMEM with 10% FCS with P/S, fixed with 3.7% formaldehyde, stained with indicated antibodies, or DAPI (4',6-diamidino-2-phenylindole, blue, Sigma), and examined using a confocal microscopy

(Nikon).

**Proliferation Assays using xCelligence** (Real-Time Cell Analyzer, Roche Applied Science) Proliferation assays were performed and analyzed by xCelligence (Real-Time Cell Analyzer, Roche Applied Science) using 293T cells transfected with indicated plasmids (Fig. 6A) by PEI method or siRNA treated HT1080 cells by CUY21 Pro-vitro (NEPA GENE Co. Ltd) (Fig. 6B).

**Cell Cycle analysis and western blot of DS cells** (Fig. 6C-6I)

The cells transfected with indicated expression vectors, harvested, fixed with 70% ethanol, stained with anti-Flag or anti-HA antibody, Alexa 488 rebelled rabbit anti-mouse IgG, and 7-AAD and analyzed.

**Antibodies and siRNA** used in this study were listed in the supplemental information.

**Statistical analysis**

Statistical analysis was verified by student T test, and  $p < 0.05$  considered as statistically significant.

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**Figure 1. TTC3 specifically associates with Akt in mammalian cells.**

**A.** Diagram showing the structure and the functional domains of Flag-tagged wild-type and TTC3 subfragments in mammalian expression vectors (N-Term, Int-, and C-Term TTC3) used in the current study.

**B.** Flag-tagged TTC3 interacted with HA-tagged Akt1 (lane 1), Akt2 (lane 4), and Akt3 (lane 7) in a co-immunoprecipitation assay. Similar amounts of each Akt isoform were immunoprecipitated by HA antibody.

**C.** Using Hs52.Sk cells, Down syndrome-derived skin fibroblast, in which TTC3 is endogenously overexpressed, TTC3 interacted with endogenous Akt1, Akt2, and Akt3.

**D.** Specificities of the Akt-TTC3 interaction were demonstrated, since TTC3 did not interact with PDK1 or PrKA by using transfected 293T cells (lanes 4-6 and 7-9). Similar levels of Akt, PDK1, and PrKA were immunoprecipitated by HA antibody. Flag-TTC3 expression was shown underneath as internal controls (HA=anti-HA

antibody; F=anti-Flag antibody; C=Control antibody).

**E.** and **F.** To define the domains of TTC3 that interact with Akt, a series of Flag-tagged TTC3 subfragments (panel **E**) and HA-Akt (panel **F**) were generated. Amino acids from 491 to 1539 of TTC3 (Int-TTC3) were the binding domain for Akt in co-immunoprecipitation assays (panel **E**, lanes 7-9, Int-TTC3). The C-terminal of Akt is required for TTC3 interaction (panel **F**, lanes 7-9).

**Figure 2. TTC3 is an E3 ubiquitin ligase for Akt.**

**A.** Since TTC3 contains a canonical E3 RING-finger motif, we first determined if TTC3

actually has an ubiquitin ligase activity. An *in vitro* ubiquitination assay was performed using recombinant TTC3 (see **Fig. S1**) together with [recombinant rabbit E1, recombinant human E2s, phosphocreatine kinase, and bovine ubiquitin] and immunoblotted with anti-ubiquitin antibody. TTC3 was polyubiquitinated only in the presence of Ubc4 or UbcH5C as E2 enzymes (panel **A**, lanes 5 and 8; Ubc4 and Ubc5C, respectively). Ubiquitin ligase CHIP protein with Ubc5C was used as a positive control (PC).

**B.** Next, we performed an *In vitro* ubiquitination assay to evaluate the requirement of ATP, ubiquitin (Ub) and E2 (Ubc5C) for the TTC3ubiquitination activity. Efficient ubiquitination were achieved only in the presence of TTC3 as an E3 ligase, but lack of any other components (Ubc5C, ubiquitin, or ATP) achieved non-detectable levels of ubiquitination (lane 3).

**C.** In *in vitro* Akt ubiquitination assays, TTC3 ubiquitinated the phosphorylated Akt

much more efficiently than the non-phosphorylated Akt (compare lanes 7 and 4, active Akt vs. unactive Akt, respectively). Equal amounts of Akt were used, shown by immunoblotting in the lower panel. The levels of Akt phosphorylation were verified by immunoblotting as shown in Fig. 3B.

**D.** Left panel: Efficient Akt polyubiquitination was induced only in the cells transfected with Flag-TTC3 and HA-Akt with His-ubiquitin (lane 4). HA-Akt (HA) or TTC3 expressions after Immunoprecipitation were shown in the lower panel (lanes 3-4). Right panel: TTC3-induced ubiquitination was Akt-specific, since TTC3 solely induced ubiquitination of Akt (lane 7), but not AK1 kinase (lane 6). The HA-Akt and Flag-TTC3 expression after immunoprecipitation are shown (lower panels)

**E.** Wild type TTC3 or  $\Delta$ RF-TTC3 (lacks ring finger domain) with pCGN-HA-Ub (HA-tagged ubiquitin) were transfected into 293T cells. It is clear that the C-terminal RING-finger motif of TTC3 was required for efficient polyubiquitination of Akt. An equal amount of Akt was immunoprecipitated, shown by Akt immunoblot. The expressions of TTC3 (Flag) are shown by immunoblot (lower panel).

**F.** TTC3 conjugated with K48-linked polyubiquitin chains on Akt, since only wild-type ubiquitin, but not K48R-ubiquitin, induced efficient ubiquitination. The expressions of Akt after immunoprecipitation and the expression of TTC3 (Flag) are shown (lower panels).

**G.** Suppression of endogenous TTC3 expression by siRNA (top panel) completely abolished efficient Akt ubiquitination [middle panel, TTC3-siRNA (lane 2) or control siRNA treated cells (lane 4), respectively]. Equal amounts of Akt were immunoprecipitated (lower panel).

**H.** An in vivo ubiquitin assays, TTC3 preferentially ubiquitinated with wild type Akt, but not T308A-S473A-Akt2, in which both threonine 308 and serine 473 were substituted into alanine. Equal amounts of immunoprecipitated Akt or Flag-TTC3 expression were verified by immunoblotting (lower panels).

**Figure 3. TTC3 preferentially binds to phosphorylated Akt and facilitates proteasomal degradation.**

**A.** NIH3T3 cells were cultured in the presence (or absence) of FBS to activate (or inactivate) Akt. The interaction of endogenous TTC3 with Akt was enhanced by serum stimulation, which induced Akt activation (upper panel). Levels of expression of phospho-Thr308Akt, pan-Akt, and endogenous TTC3 are also shown.

**B.** In GST pull-down assays were performed using active and unactive Akt (Upstate Biotech, USA), recombinant TTC3. Active Akt, but not unactive Akt, interacted efficiently with GST-TTC3 (left panel, lanes 3 and 4, active- and unactive-Akt, respectively). Akt phosphorylations at Ser473 or Thr308 were confirmed by immunoblotting (right panels).

**C.** 293T cells were transfected with wild type TTC3 with pCGN-HA-Ub. PDGF stimulation, known to activate Akt, induced efficient Akt polyubiquitination (upper panel). Levels of Akt expression (second panel) and phospho-Thr308Akt (third panel) after immunoprecipitation are shown.

**D.** In co-immunoprecipitation assays TTC3 preferentially interacted with wild type Akt, but not T308A-S473A-Akt2, in which both threonine at 308 and serine at 473, critical residues for Akt activation, were substituted into alanine. The levels of Akt and TTC3 are shown in the lower panels.

**E.** TTC3 expression was reduced by TTC3 siRNA in a dose-dependent manner (top panel), which was correlated inversely with phosphorylated Akt levels (phospho-Thr308 and phospho-Ser473, 2<sup>nd</sup> and 3<sup>rd</sup> panel from the top, respectively). Increased phosphorylated Akt expression was correlated with that of phosphorylation of FKHR (forkhead transcriptional factor, 6<sup>th</sup> panel), without affecting the amount of total FKHR (5<sup>th</sup> panel). TTC3 siRNA did not affect the levels of MAP kinase (7<sup>th</sup> and 8<sup>th</sup> panels). Control siRNA at the maximum concentration (64nM) showed no effect.

**F.** 293T cells were transfected with indicated plasmids [wild type TTC3 with or without Myr- $\Delta$ PH-Akt (Kohn et al., 1996)] by calcium phosphate method. Cells were treated with 17AAG (Basso et al., 2002) for 12 hours before CHX treatment (at time 0) with (or without) MG132. Wild type TTC3 induced Akt degradation in a

time-dependent manner in the presence of Myr-Akt, which was efficiently inhibited by MG132. Note that Myr-Akt also showed time dependent decrease in the absence of MG132 (second panel). Myr-Akt migrated at approximately 50KD on SDS-PAGE gel (also see Fig. 4D).

**Figure 4. Phosphorylation of TTC3 at Ser 378 is required for efficient biological function.**

**A.** 293T cells were transfected with indicated plasmids with (or without) Myr-Akt (Upstate #21-151). Akt activation efficiently induced phosphorylation of wild-type TTC3 (top panel, lane 5), but failed to phosphorylate S378A-TTC3 by Myr-Akt. Note that in the absence of Myr-Akt (right panels), no phosphorylation of wild-type TTC3 was observed. The expression of TTC3 after immunoprecipitation (second panel) and phospho-Ser473Akt (third panel) are also shown.

**B.** *In vitro* Akt kinase assays were performed using recombinant wild type TTC3 and S378A-TTC3. Akt efficiently phosphorylated wild type TTC3, but not S378A TTC3 (compare lanes 3 and 6, wild type TTC3 and S378A TTC3, respectively). Only Akt, but no other kinases were present in this assay, supported that Akt could phosphorylate TTC3 at Ser378.

**C.** We next performed an Akt-siRNA experiment to suppress the endogenous expression of Akt. The levels of the phosphorylation of TTC3 detected by anti-Akt substrate antibody (Cell Signaling # 9611) was diminished its intensity, supported

that endogenous Akt phosphorylated TTC3 in the cellular context, although we cannot rule out that other kinases might also be involved *in vivo*.

**D.** 293T cells were transfected with wild type human TTC3,  $\Delta$ RF-TTC3(lacks C-terminal ring finger domain), or S378A-TTC3 with pCGN-HA-Ub (HA-tagged ubiquitin). The results supported that S378 and RF of TTC3 were required for efficient Akt ubiquitination. An equal amount of Akt expression after immunoprecipitation (middle panel, lanes 1-4) and Flag-TTC3 (bottom panel) were verified by immunoblot.

**E.** TTC3 induced Akt degradation in the presence of Myr-Akt to activate Akt (top panel, lanes 1-3 vs. 4-6, in the absence vs. presence of Myr-Akt, respectively). In contrast, S378A-TTC3 failed to induce Akt degradation even in the presence of Myr-Akt.

**F.** 293 T cells were transfected with vector control, wild type TTC3, or S378A-TTC3 by calcium phosphate method. Using rhodamine123 (Rho123), TTC3 significantly increased the Rho123 high cell population compared to S378A-, wild type-TTC3, or

control. [Mean Fluorescence Intensities (MFI) were 548, 162, and 379, wild type-, S378A-TTC3, or control, respectively].

**G.** In NF $\kappa$ B luciferase reporter assays (Stratagene), wild-type TTC3, but not

S378A-TTC3, failed to induce NF $\kappa$ B transactivation (relative activities were 2460 $\pm$ 330, 11200 $\pm$ 860, 4950 $\pm$ 380, wild-type, S378A-TTC3, or control, respectively).

**H.** 293T cells were transfected with wild type-, S378A-TTC3, or control. Wild

type-TTC3 inhibited proliferative responses more efficiently than S378A-TTC3 or control. Note that Myr-Akt efficiently reversed the inhibitory effects of TTC3 (see

**Fig. S4A-C).**

**Figure 5. TTC3 associates with Akt and induces proteasomal degradation of Akt within the nucleus.**

**A.** 293T cells were transfected with wild type-TTC3 and HA-Ub. Endogenous Akt (panel A) were ubiquitinated more efficiently in the nucleus (N) than in the cytoplasm(C) (compare lanes 7 and 8; cytoplasmic and nuclear fraction, respectively). Please note that nuclear Akt was highly phosphorylated compared to the cytoplasmic Akt (bottom panel, lane 4 and 8).

**B and C.** 293T cells were transfected with wild type TTC3 and treated with 17AAG and CHX (at time 0 for degradation assay) in the presence (or absence) of MG132. Cells were then harvested at indicated time points, cytoplasmic and nuclear fractions were separated. TTC3 facilitated Akt degradation in the nucleus more efficiently than in the cytoplasm (compare upper panel, cytoplasmic in lanes 1-3 vs. nuclear fraction in lanes 4-6, respectively). MG132 treatment efficiently inhibited Akt degradation (second panel). Times (hrs) are shown after CHX treatment (time 0). Relative intensities were measured using NIH Image J (panel

C). Note that control transfected cells showed minimal Akt degradation in the nuclear fraction (data not shown).

**D.** In NIH3T3 cells endogenous TTC3 (green) co-localized with Akt (red) predominantly within the nucleus during the interphase of cell cycle by using a confocal microscopy (top row). Akt co-localized with TTC3 around the nuclear membrane at prophase of the mitotic cells (second row, right panel, indicated by white arrow). During the later period of the mitotic phase, TTC3 and Akt became tightly co-localized with each other around the kinetochore and midzone of the cell.

**E - F.** Further, TTC3 (green) co-localized with phosphorylated Akt (Ser-473, red) both interphase (**E**) and in the mitotic phase (**F**) of the cell cycle.

**G.** In Hs52.Sk cells from DS, in which TTC3 is overexpressed (see Fig. 6C), endogenous TTC3 co-localized with Akt in the nucleus.

**Figure 6. Functional interaction of Akt-TTC3 in Down Syndrome cells**

**A.** Proliferation assays were performed and analyzed by xCelligence (Real-Time Cell Analyzer, Roche Applied Science). Wild-type TTC3 significantly inhibited proliferative responses. The results were consistent in three independent experiments.

**B.** HT1080 cells were treated with TTC3-siRNAs or control scrambled RNA (IDT) by CUY21 Pro-vitro (NEPA GENE Co. Ltd). Proliferative responses were enhanced by siRNA specific for TTC3 (four different targeting siRNA for TTC3 designated as #1-#4, indicated by closed triangles, squares, x, or circles, respectively, see supplemental information), compared to the control siRNA (indicated by open circles), analyzed by xCelligence (Real-Time Cell Analyzer, Roche Applied Science).

**C.** Hs52.Sk and CMK85 cells from DS, with high endogenous TTC3 expression, exhibited inverse correlations between TTC3 and phosphorylated Akt at Thr-308 expression.

- D.** CMK85 cells were treated with indicated concentration of TTC3-siRNA or control siRNA and analyzed using CUY21 Pro-vitro (NEPA GENE Co. Ltd). TTC3 siRNA reduced TTC3 expression (top panel), which was correlated inversely with phosphorylated Akt at Thr-308 in a dose dependent manner (middle panel).
- E.** 293T cells were transfected with TTC3 or control by PEI. TTC3 suppressed the G<sub>2</sub>M to G<sub>1</sub> transition; hence it increased the relative number of G<sub>2</sub>M cells. Relative accumulation of cells in G<sub>2</sub>M to G<sub>1</sub> transition shown by G<sub>1</sub>/G<sub>2</sub>M ratio was decreased by 70% compared to control. Relative ratio of G<sub>1</sub>/G<sub>2</sub>M is shown as a bar graph (panels E-I).
- F. and G.** Ectopic expression of TTC3 into HDF cells (**F**) or Jurkat cells (**G**) suppressed the G<sub>1</sub>/G<sub>2</sub>M ratio by over 70% gated on the TTC3 (Flag) positive cell. Hs52.Sk or CMK85 cells, with high endogenous TTC3 expression, showed reduced G<sub>1</sub>/G<sub>2</sub>M ratio by 80% compared to control cells.

**H.** TTC3-siRNA or control siRNA with pCMV-EGFP were transfected into CMK85 cells. The cells were, fixed, stained with 7-AAD, and analyzed by FACS.

Introduction of TTC3-specific siRNA into CMK85 cells overcame the G<sub>2</sub>M phase; hence an increase in the G<sub>1</sub>/G<sub>2</sub>M ratio by more than 400% compared to the control gated on the GFP expressed cells.

**I.** CMK85 cells transfected with HA-tagged Myr-Akt, fixed, stained with anti-HA antibody with 7-AAD. Introduction of Myr-Akt overcame the G<sub>2</sub>M phase checkpoint transition, hence it exhibited more than a 300% increase in the G<sub>1</sub>/G<sub>2</sub>M ratio gated on the Myr-Akt expressed cells.

**Figure 7. TTC3 is a novel E3 ubiquitin ligase for Akt**

Akt, a core intracellular survival regulator, is known to be activated at the plasma membrane and subsequently translocated to the nucleus. However, little is known about the silencing mechanisms of activated Akt after nuclear translocation. Based on the current study, TTC3 is a novel E3 ubiquitin ligase for Akt that preferentially binds to phosphorylated Akt, hence facilitating ubiquitination and proteasomal degradation within the nucleus. Since TTC3 is located within the DSCR of human Chromosome 21, the current study will provide not only the biological significances of the TTC3-Akt functional interaction, but also a new insight for the molecular mechanisms of DS, the most common genetic disorder in humans.

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Fig. 1

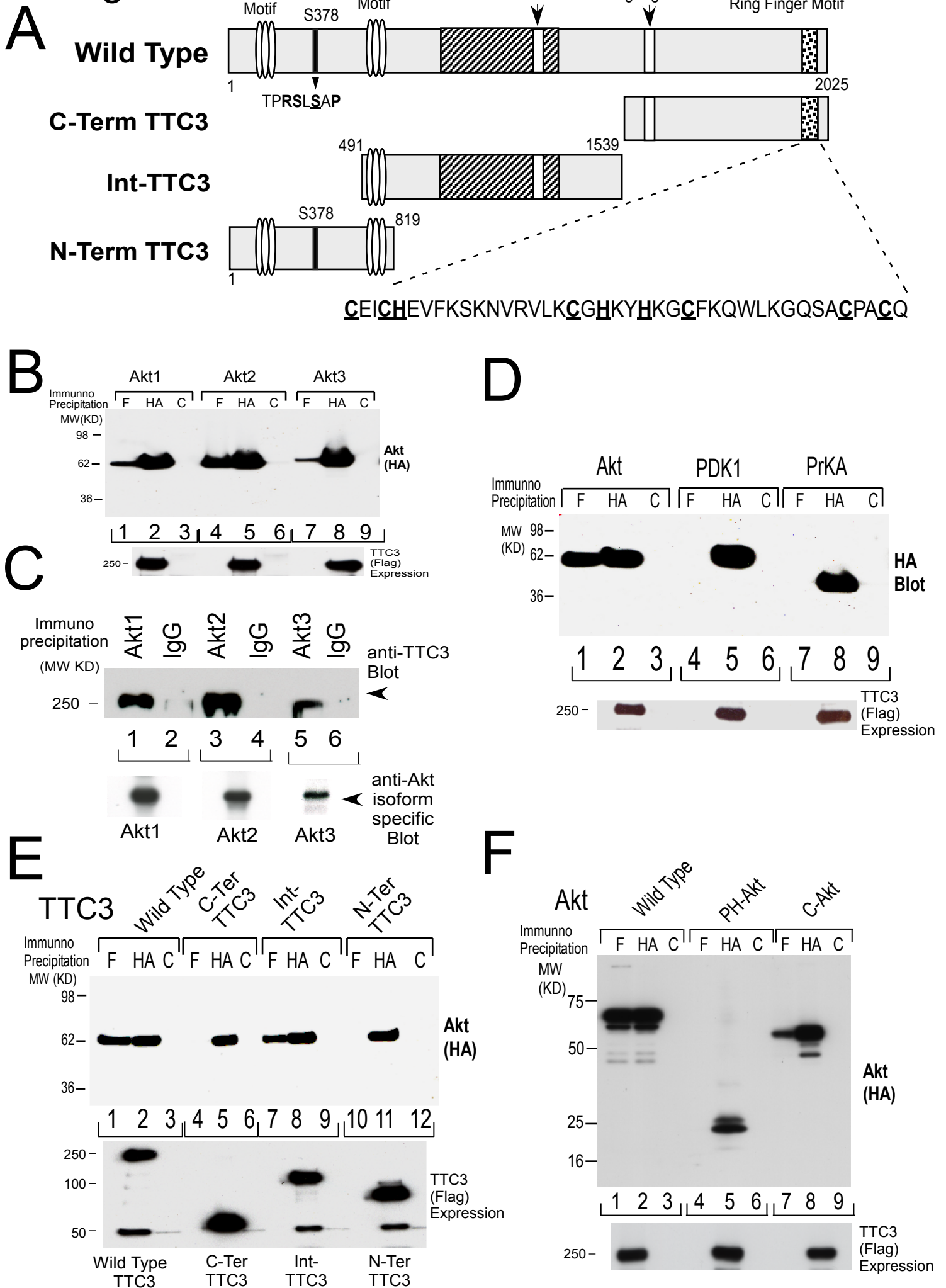
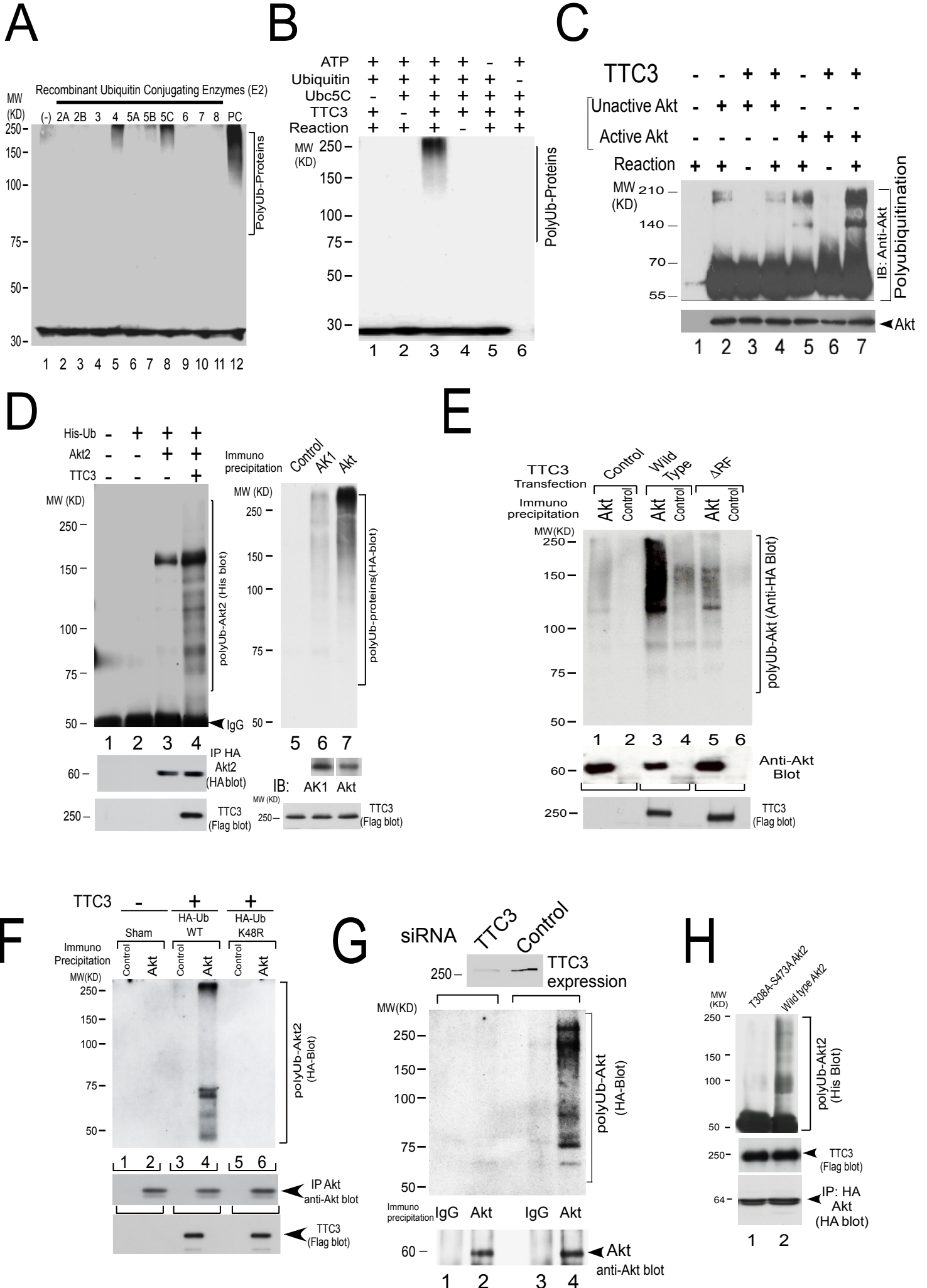
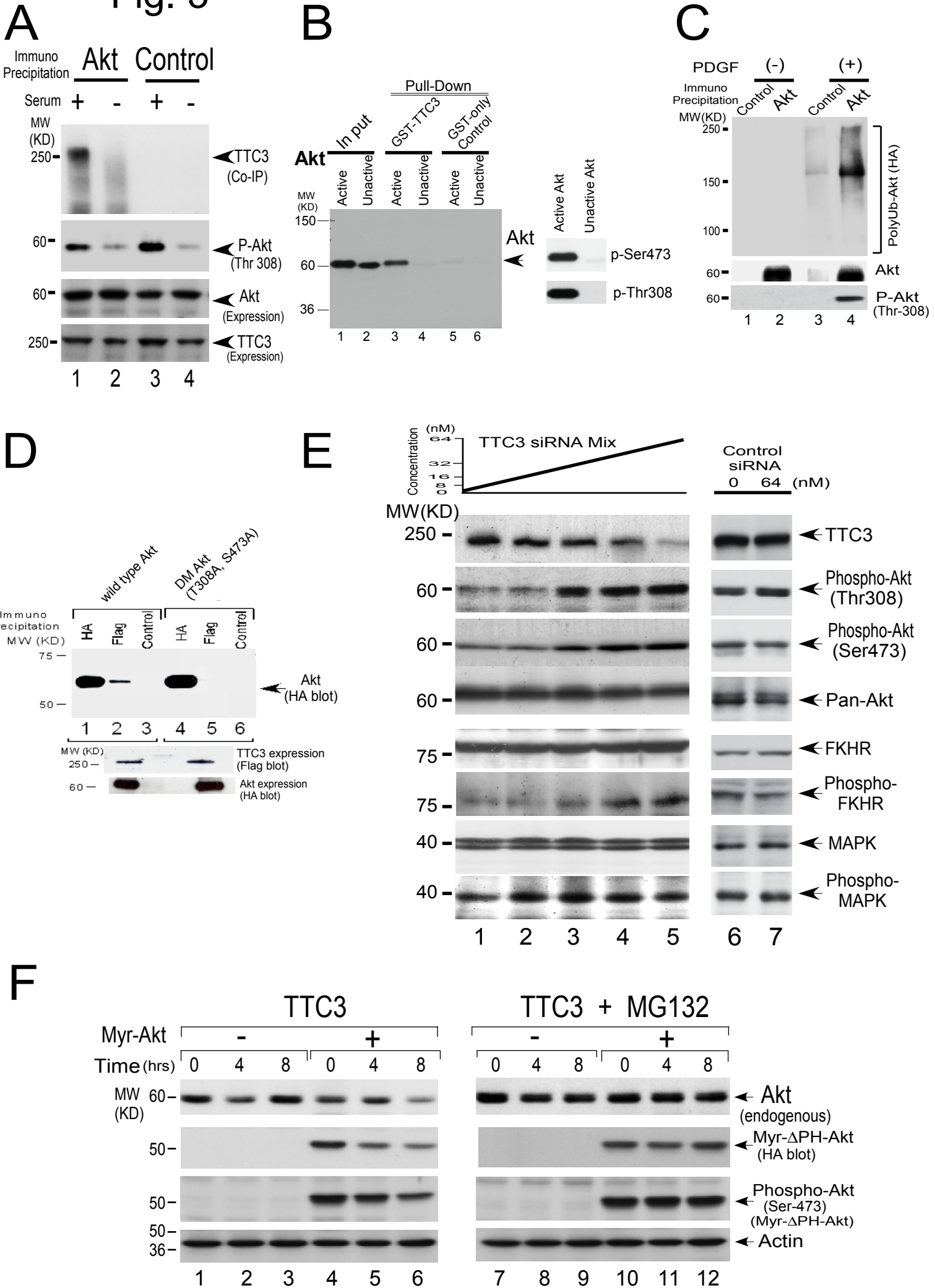


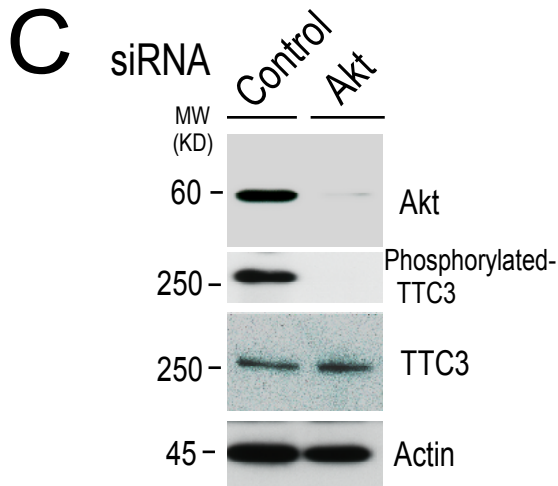
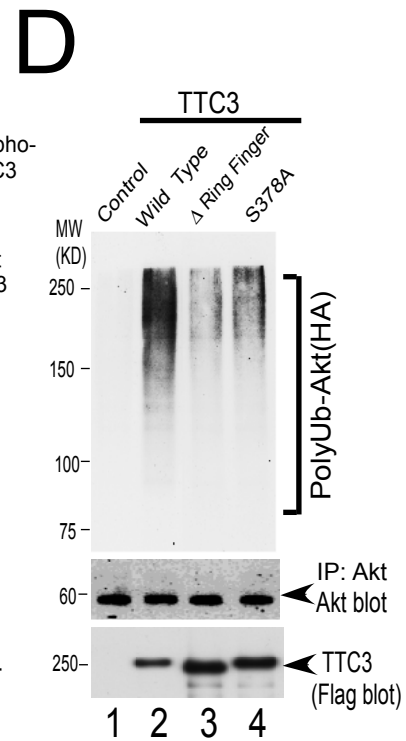
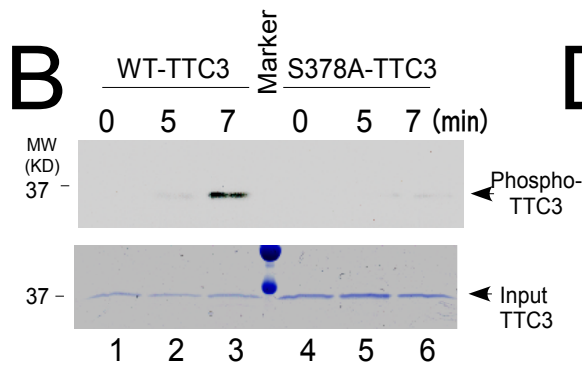
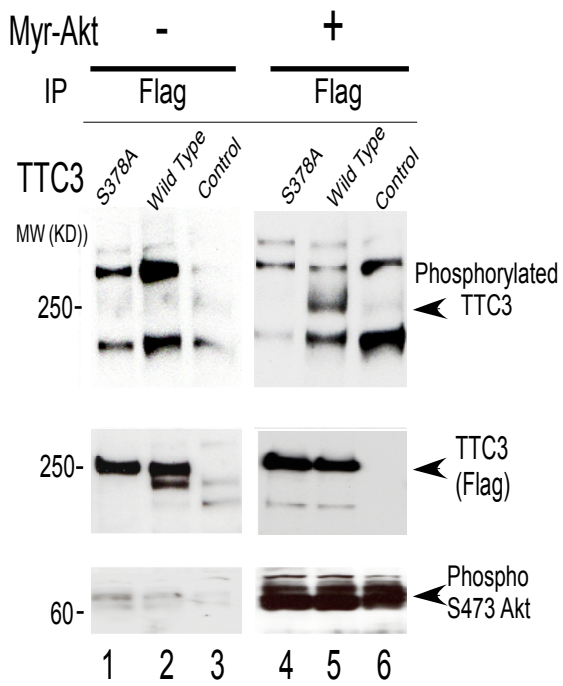
Fig. 2



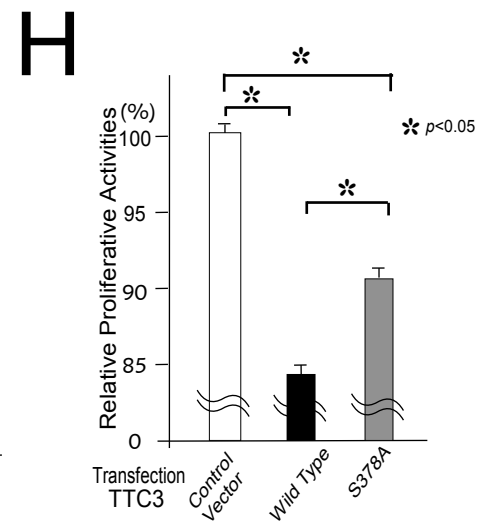
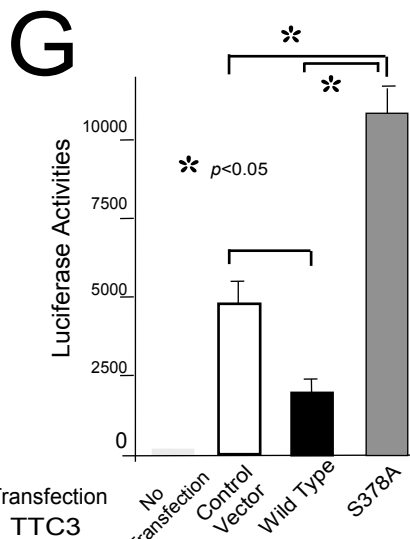
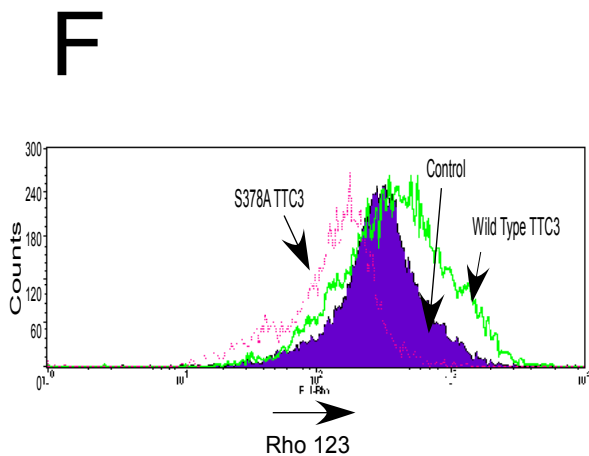
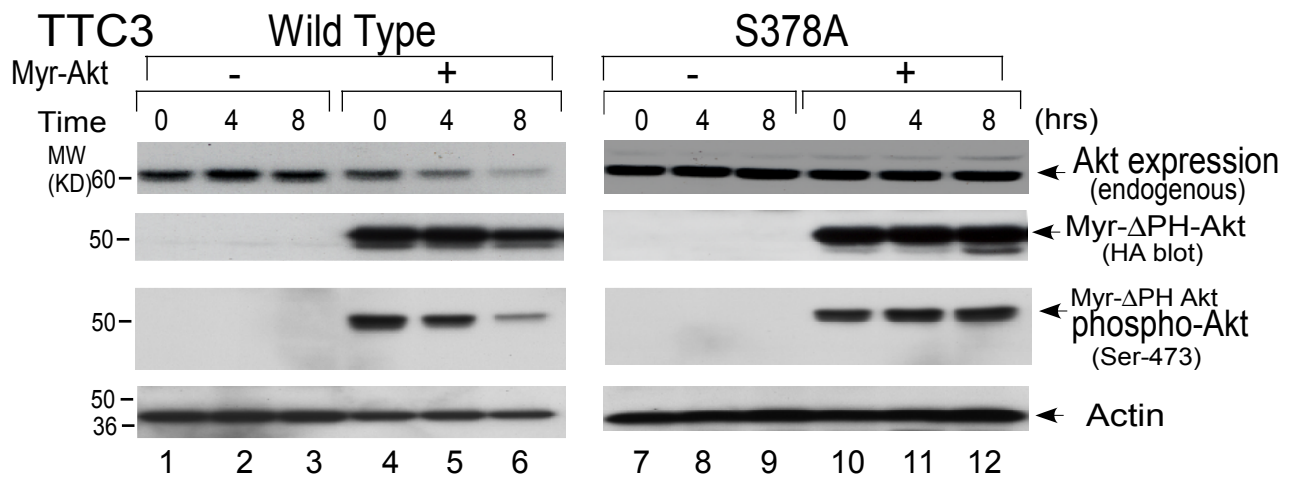
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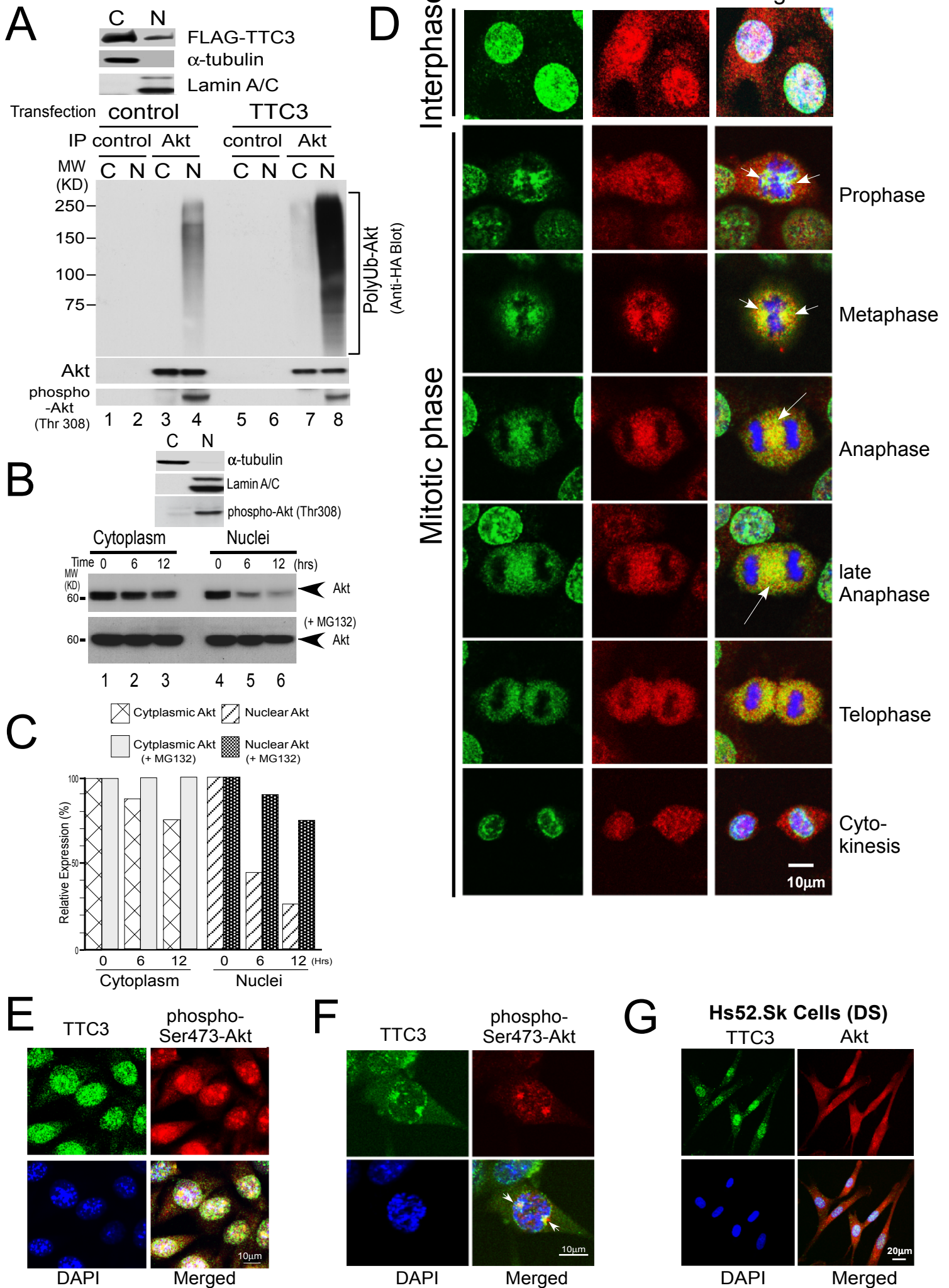
# A Fig. 4



# E

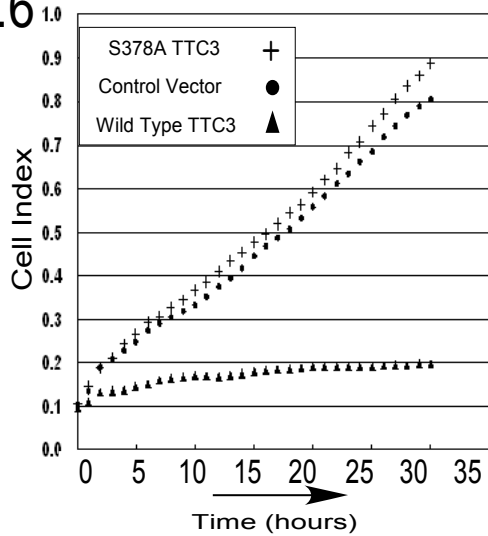


**Fig. 5**

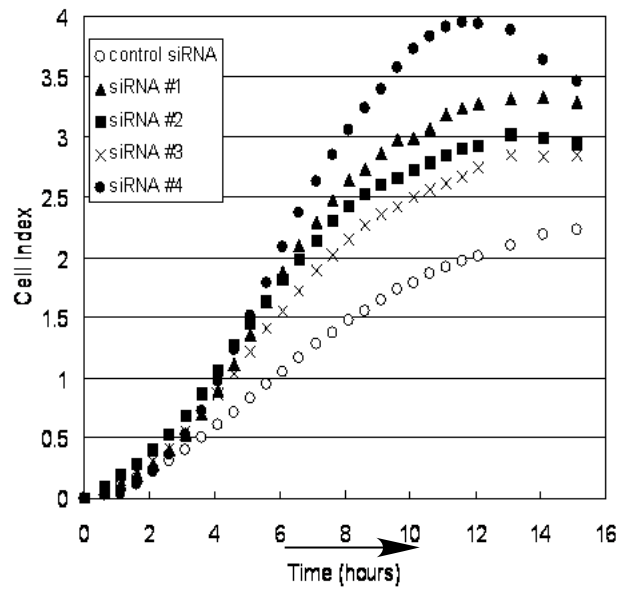


**Fig.6**

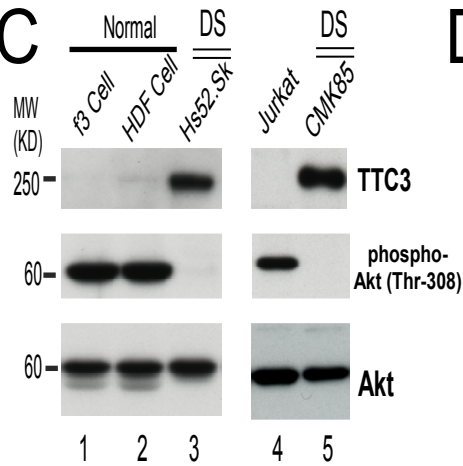
**A**



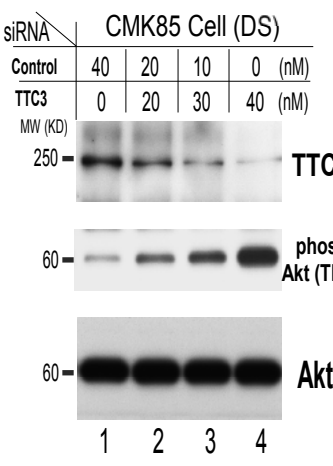
**B**



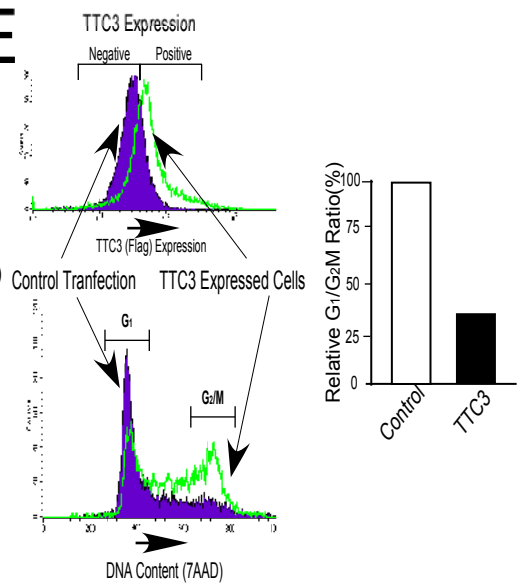
**C**



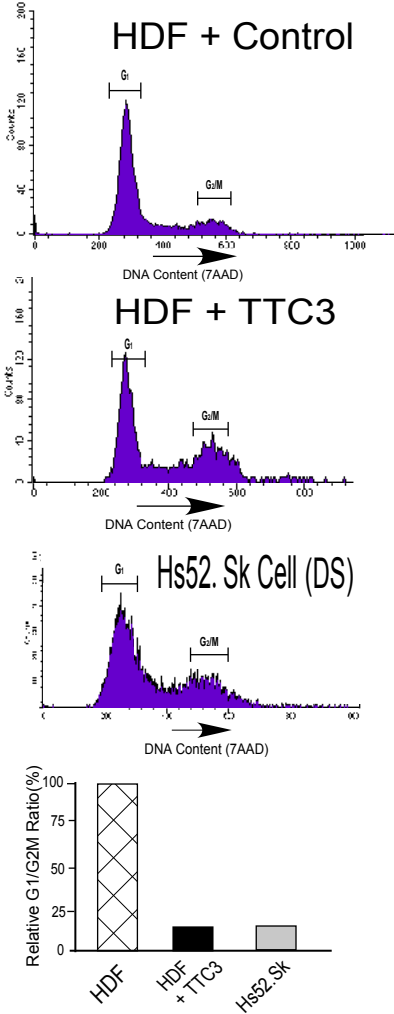
**D**



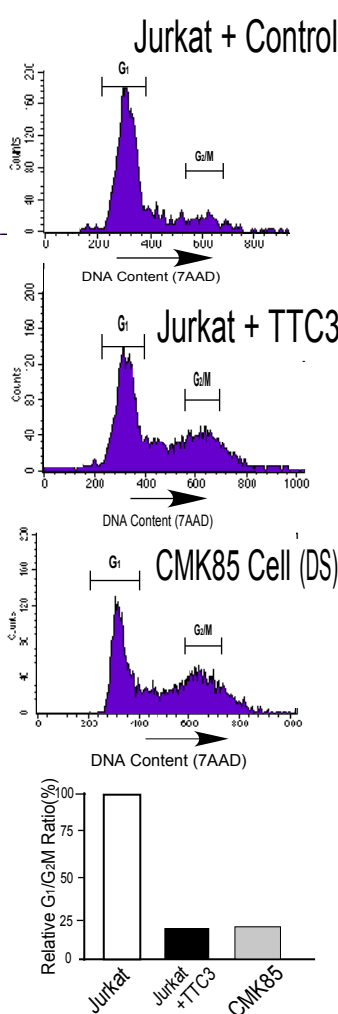
**E**



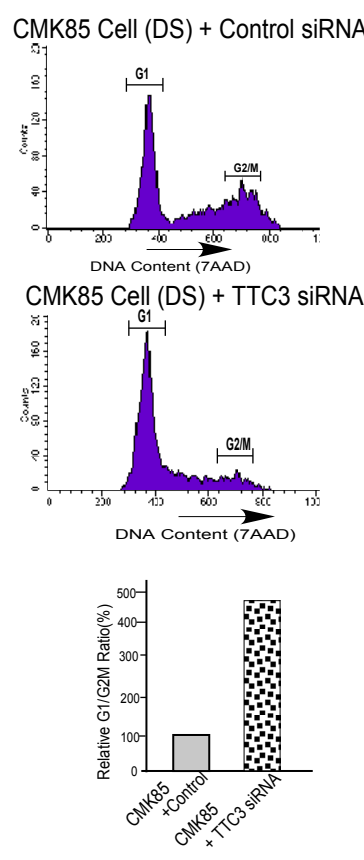
**F**



**G**



**H**



**I**

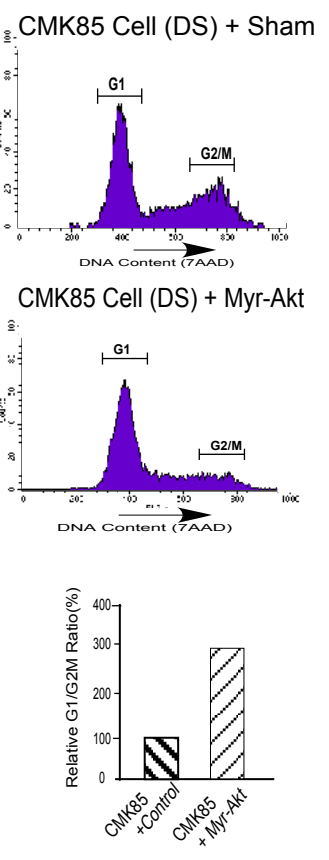
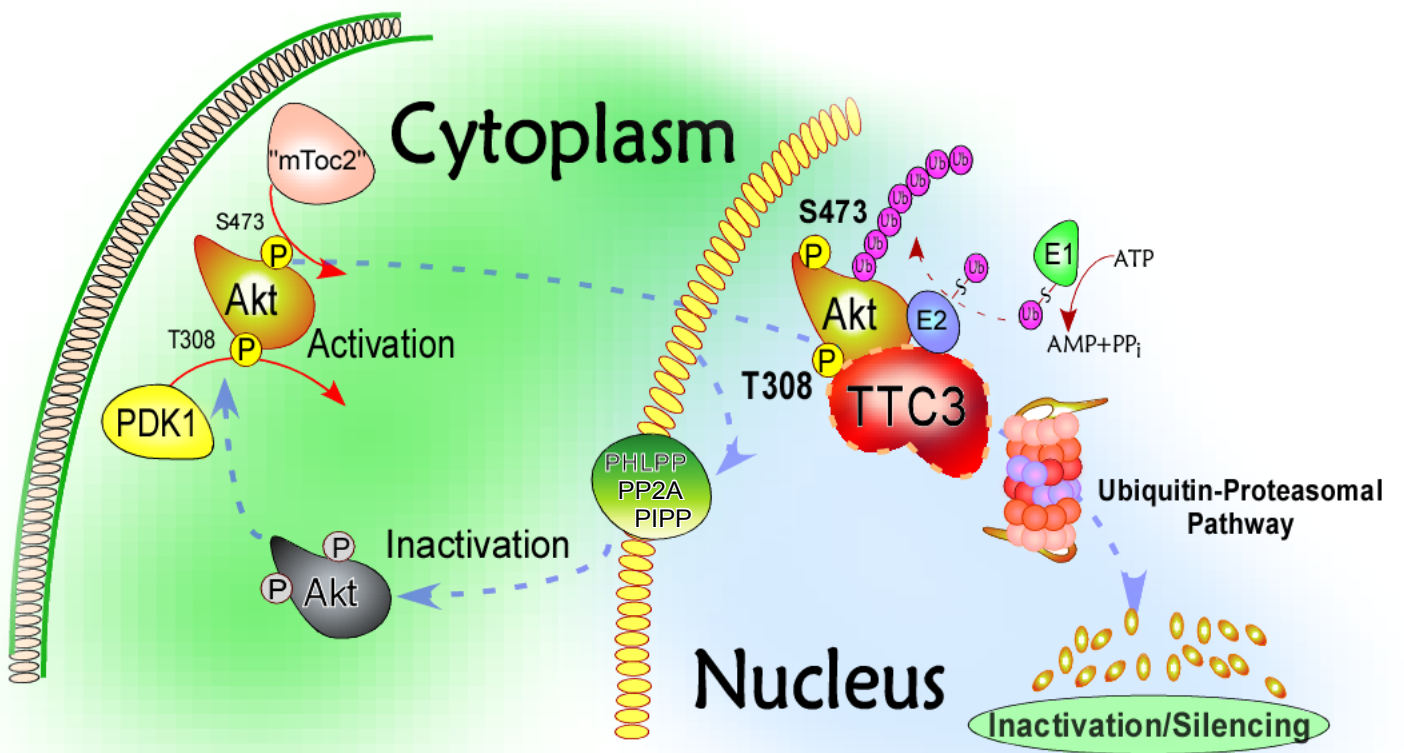


Fig. 7

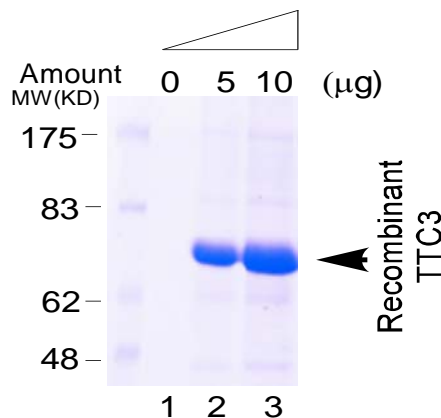


## Supplemental Information

### *Supplemental data:*

#### Supplemental data Fig. S1.

#### *Generation and Purification of Recombinant TTC3*

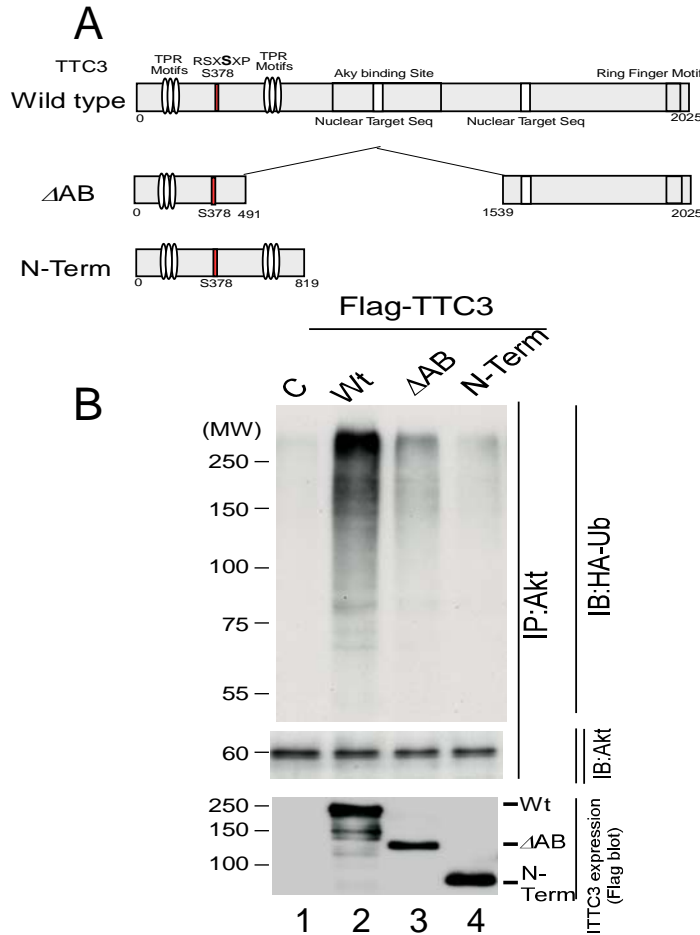


The purified recombinant products of TTC3 used for in vitro ubiquitin assays (Fig. 2A and 2B) were shown by Coomassie staining of the SDS-PAGE. Although the predicted molecular weight of the recombinant TTC3 (Amino Acid from 1540 to 2025 of human TTC3) was 55KD, the resulted recombinant proteins were migrated at approximately 70KD on SDS-PAGE because of possibly altered posttranslational modification.

**Method:** Recombinant TTC3 was generated by pFastBac HTC baculoviral system (Invitrogen) essentially described previously (Laine et al., 2000). The subfragment of human TTC3 (Amino Acid from 1540 to 2025 of human TTC3) was amplified by PCR and subcloned into pFastBac HTC (Invitrogen). The resulted plasmid was co-transfected with Baculo-gold DNA into Sf-9 cells using Baculogold kit (PharMingen). Sf-9 cells were harvested after 60 hours, lysed with the following lysis buffer [50mM Tris-HCl buffer (pH 7.5), 1% Nonidet P-40, 10% glycerol, 137mM NaCl, 50mM  $\beta$ -glycerophosphate, 4 $\mu$ g/ml AEBSF, 4 $\mu$ g/ml aprotinin, 4 $\mu$ g/ml pepstatin A, and 4  $\mu$ g/ml leupeptin], and spun at 15,000 x g for 30min. The His x 6-tagged TTC3 was purified using a Ni<sup>2+</sup>-chelating affinity column after extensive washing using LiCl<sub>2</sub> washing buffer (lysis buffer plus 20mM imidazole and 0.5M LiCl) and eluted with elution buffer (lysis buffer plus 0.5M imidazole). The purity of the final purified recombinant TTC3 products was verified by Coomassie staining of the SDS-PAGE.

**Supplemental data Fig. S2.**

***TTC3-Akt interaction is important for efficient Akt ubiquitination***



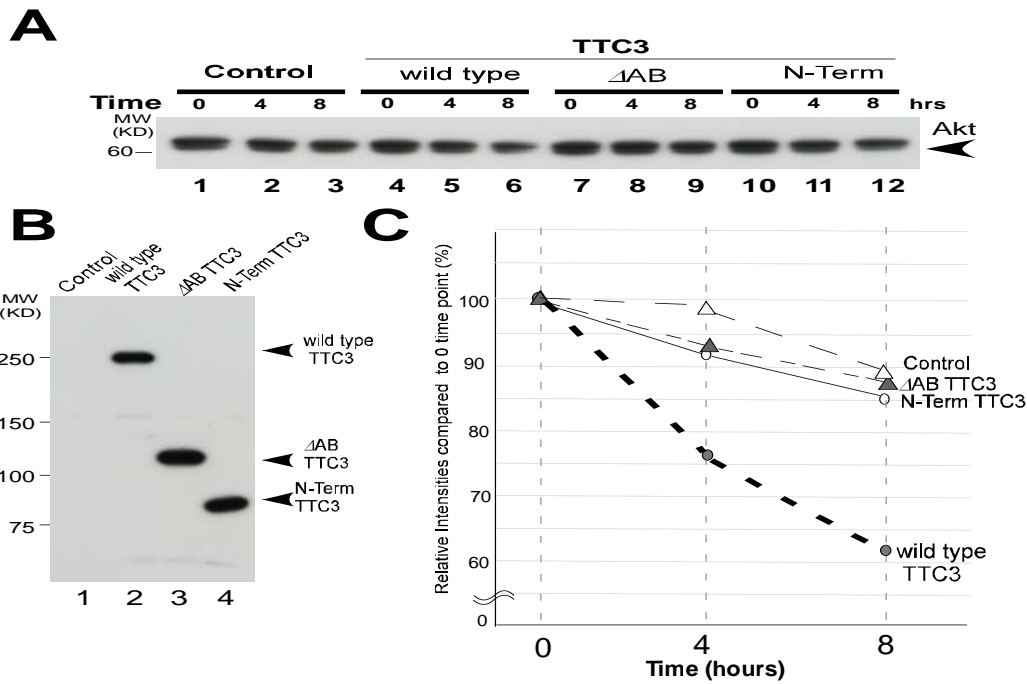
**A.** Diagrams of TTC3 using in the study were shown. We generated  $\Delta$ AB-TTC3 (consisting of AA 1~491 and 1539~2025 of TTC3) or N-terminal TTC3 (consisting of AA 1~819 of the N-terminal TTC3), both of which lack the putative Akt-binding domain, to compare the efficiencies of ubiquitination of Akt in the cellular context.

**B.** Compared to wild type TTC3 transfected cells, significantly low levels of Akt ubiquitination were achieved by either  $\Delta$ AB-TTC3 or N-termTTC3 (see Fig. S2), suggesting the importance of the binding for Akt

ubiquitination. Levels of Akt expression after immunoprecipitation and Flag-TTC3 were shown in the 2<sup>nd</sup> and 3<sup>rd</sup> panels.

**Method:** Plasmid vector containing a partial cDNA fragment of Flag-tagged human TTC3 generated by PCR or by the restriction digests. Human 293T cell lines were transfected with indicated TTC3 constructs (wild type,  $\Delta$ AB, or N-Term TTC3) along with and HA-ubiquitin. Fifteen hours after transfection, the cells were treated with 10 $\mu$ M MG132 (Sigma) for 6hrs, rinsed with PBS, and lysed with Brij97 lysis buffer (Laine et al., 2000) with protease inhibitors, 10 $\mu$ M MG132, and 5mM iodoacetamide (Hatakeyama et al., 2001; Lorick et al., 1999). The cell lysates were precleared, immunoprecipitated with anti-Akt antibody, and the samples were resolved onto SDS-PAGE, immunoblotted, and detected by ECL.

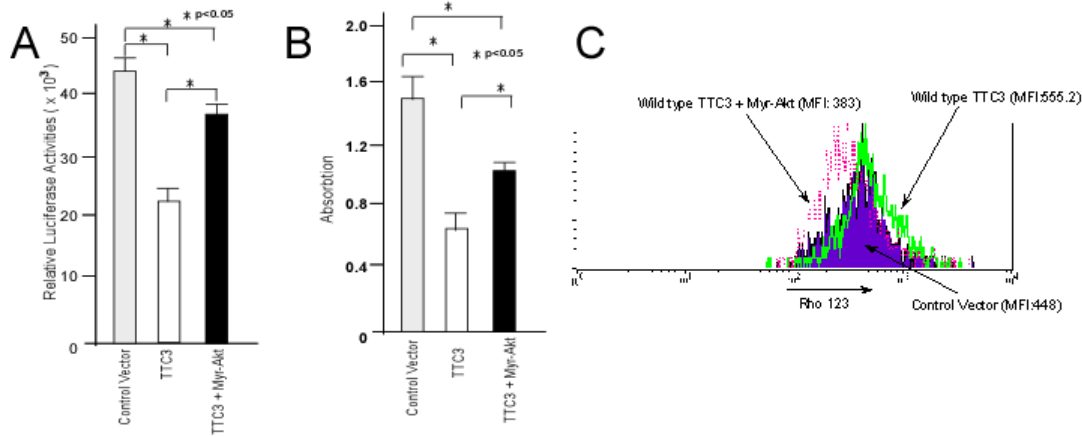
Supplemental data Fig. S3. *TTC3-Akt interaction and degradation of Akt*



**A.** Akt degradation of 293T cells transfected with  $\Delta$ AB-TTC3 or N-term TTC3 (see Fig. S2), which lacked for Akt binding abilities, were clearly more compromised than wild type TTC3 transfected cells, as determined by Akt immunoblot. **B.** Expressions of Flag TTC3 (wild type,  $\Delta$ AB-TTC3, or N-Term TTC3) were shown by immunoblot. **C.** Relative intensities of Akt at each time point after CHX treatment (time 0 for degradation assay) at 8 hours after CHX treatment were 63% (wild type TTC3), 88%, ( $\Delta$ AB-TTC3), 86% (N-Term TTC3), or 89% (control) after being normalized by expressions of Flag TTC3. Shown in panel B.

**Method:** 293T cells were transfected with indicated plasmids (control vector, wild type TTC3,  $\Delta$ AB-TTC3, or N-Term TTC3) by calcium phosphate method, 24 hours later, cells were treated with 10 $\mu$ M 17AAG (Basso et al., 2002) for 12 hours before 100 $\mu$ g/ml CHX treatment (time 0). Cells were harvested at indicated time points after CHX treatment (time 0, 4, and 8 hours), lysed with Brij 97 lysis buffer in the presence of 1mM Na<sub>3</sub>VO<sub>4</sub>, 10mM NaF, 10 $\mu$ M MG132, and 5mM iodoacetamide (Hatakeyama et al., 2001; Lorick et al., 1999). Nuclear fractions were separated by hypotonic and hypertonic method described, resolved onto SDS-PAGE, and immunoblotted using ECL. Expression of each TTC3 construct was shown by Flag immunoblotting used for internal control. Quantitation of the signal at time 0, 4 and 8 hours after CHX treatment were calculated using NIH Image J after normalized by the expression of Flag-TTC3 (panels B and C).

**Supplemental data Fig. S4. *Myr-Akt* reverted the biological consequence of *TTC3***

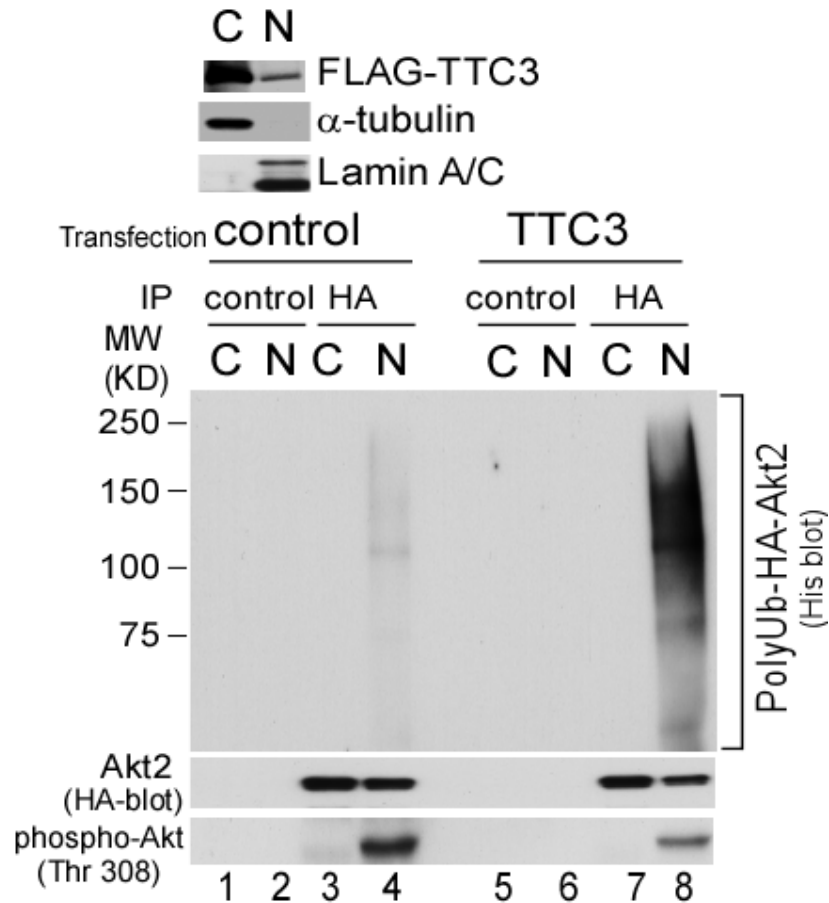


**A.** Inhibitory effect of the NF $\kappa$ B Luciferase reporter activity could be reverted by the co-introduction Myr-Akt, a constitutive active form of Akt. The results were from triplicate value and were normalized by protein concentration. **B.** Myr-Akt also reverted TTC3 induced inhibition of proliferative responses of natural growing HEK293 cells. The results were from the triplicate value. **C.** Myr-Akt also reversed the effect on mitochondrial membrane depolarization induced by wild type TTC3 measured by Rho123 staining. Mean fluorescence intensities (MFI) were 555.2 vs. 383, wild type TTC3 vs. wild type TTC3 + Myr-Akt transfected cells, respectively.

**Method:** Luciferase reporter assays were essentially performed described elsewhere (Hiromura et al., 2006) using Luciferase kit (Promega). 293T cells (ATCC) were cultured in the presence of 10% FCS in DMEM and transfected 1.5 $\mu$ g/12 well dish of the indicated plasmid (control vector, wild type TTC3, wild type TTC3 with Myr-Akt, [HA-Myr- $\Delta$ PH(4-129) human-Akt1 in pECE vector (Kohn et al., 1996)]) along with 100 ng/ well of the luciferase NF $\kappa$ B reporter vector (Stratagene) by PEI (Polyscience Inc. #23966). Proliferation assays were performed as described. 293T cells were transfected with 3 $\mu$ g of wild type TTC3 or TTC3 + Myr-Akt in mammalian expression vectors, or control vector) by PEI method. Two days after the transfection, 1  $\times 10^3$  of the transfected cells were seeded onto 96 well plates (corning #3596). Forty hours later, 20 $\mu$ l of the Cell Titer AQueous One Solution Reagent (Promega G3580) was then added onto each well. Two hours later, the absorption (OD 490nm) was measured by ELISA methods. The values shown were relative proliferative activities compared to the vector control out of three replicates. Similar results were obtained in another complete set of the independent experiment. For, MTP assays, 293 T cells were transfected with a total of 1.5 $\mu$ g of indicated plasmids DNA/6cm dish (vector control, wild type TTC3, or wild type TTC3 with Myr-Akt) using PEI method. Forty eight hours after transfection, cells were treated with 50ng/ml of human TNF- $\alpha$  (PeproTech) for additional 2 hours at 37 $^{\circ}$ C. The cells were inoculated with 2.5 $\mu$ M Rho 123(Molecular Probes) for 15 minutes. The Rho123 fluorescence intensities were analyzed in 5 $\times 10^3$  cells/sample in combination with 2 $\mu$ g/ml propidium iodide staining (PI, Boehringer Mannheim) and analyzed gating on PI negative cells using flow cytometry (Laine et al., 2000).

**Supplemental data Fig. S5.**

***TTC3 facilitated polyubiquitination of Akt in the nucleus***

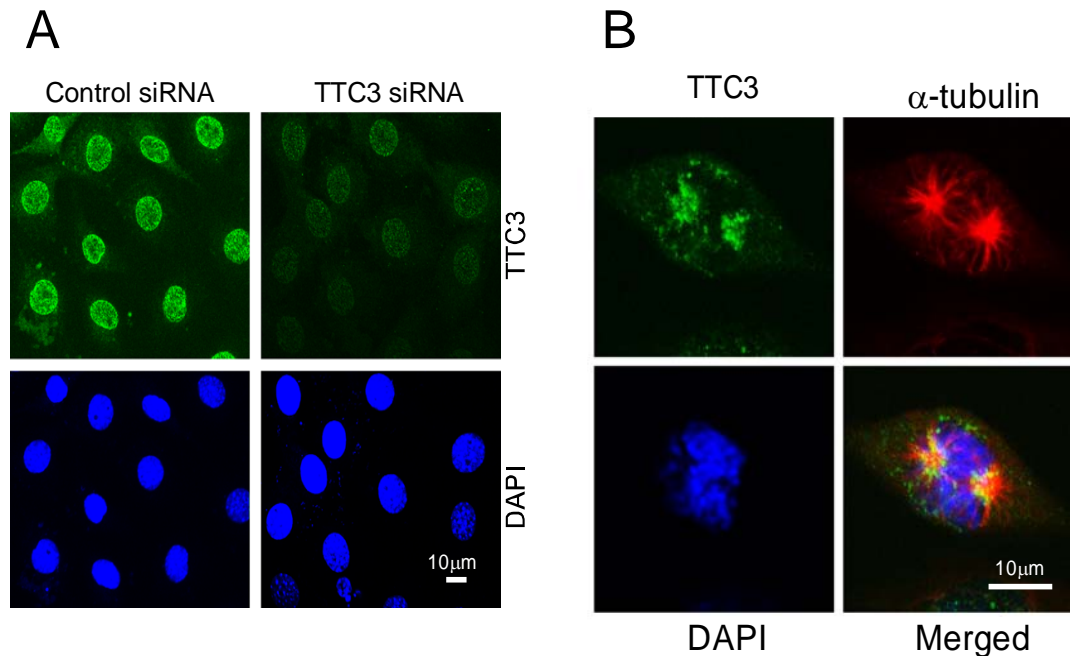


Consistent with endogenous Akt shown in Fig. 5A, ectopic transfected Akt was polyubiquitinated more efficiently in the nucleus (N) than in the cytoplasm (C) (compare lanes 7 and 8; cytoplasmic and nuclear fraction, respectively). Nuclear fraction was highly phosphorylated compared to the cytoplasmic fraction (bottom panel, lane 4 and 8).

**Method:** : The method was essentially described in Fig. 5A except 293T cells were transfected with (His-Ub and HA-Akt2) plasmids by a calcium phosphate method.

## Supplemental data Fig. S6A and B

### *Immunohistochemical analysis using anti-TTC3 rabbit antibodies*



**A.** Polyclonal anti-TTC3 rabbit antibody was generated by immunizing rabbits using peptide CQGRDLLTEESPSGRG (AA 1996-2011 of human TTC3) (Asahi Techno Glass, Japan). Specificity of the antibody against TTC3 used in these studies (Fig. 5D-F) was verified by using TTC3 specific siRNA. siRNA specific for mouse TTC3 (Invitrogen, #HSS144355, HSS144356, and HSS144357) significantly reduced the intensity of TTC3 immuno-staining, indicating that the polyclonal anti-TTC3 antibody specifically recognized endogenous TTC3 (green).

**B.** Triple merged staining of DAPI (blue), anti-TTC3 (green), and  $\alpha$ -tubulin (red) demonstrated that TTC3 was located at the kinetochore.

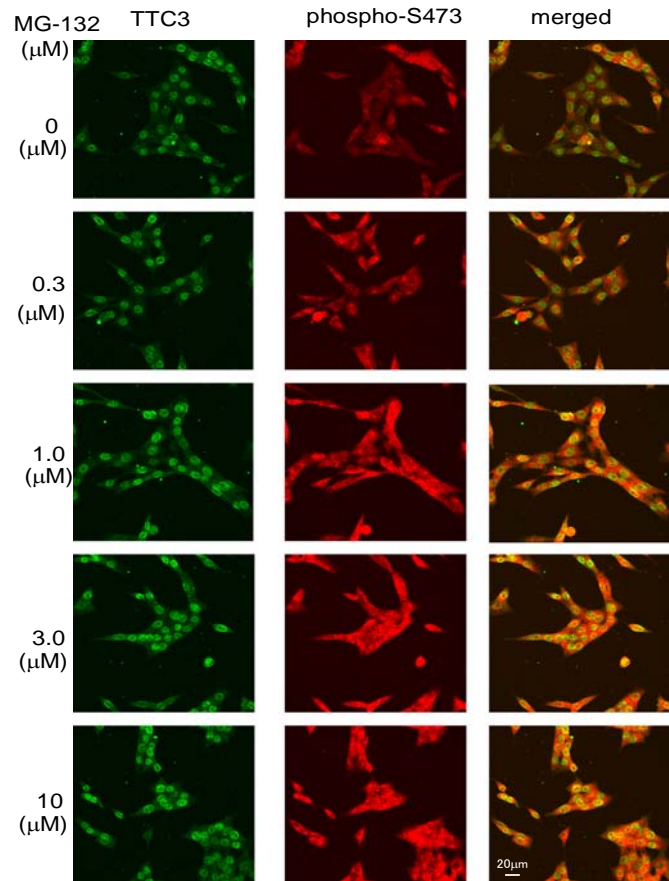
**Method :** Small interfering RNAs (siRNA), specific for mouse TTC3, were purchased from Invitrogen (#HSS144355, HSS144356, and HSS144357). Control scrambled RNAs was purchased from Integrated DNA Technologies (IDT, USA). NIH3T3 cells (ATCC) were transfected with the siRNAs at 64nM concentration by PEI (Polyscience Inc. #23966). 72 hours later cells were fixed with 3.7% formaldehyde, stained with anti-TTC3, anti- $\alpha$  tubulin, or DAPI (4', 6- diamidino-2-phenylindole, Sigma), and examined using a confocal microscope (Nikon).

**Supplemental data Fig. S7.**

***MG132 treatment enhanced co-localization of TTC3 with phosphorylated Akt in a dose dependent manner***

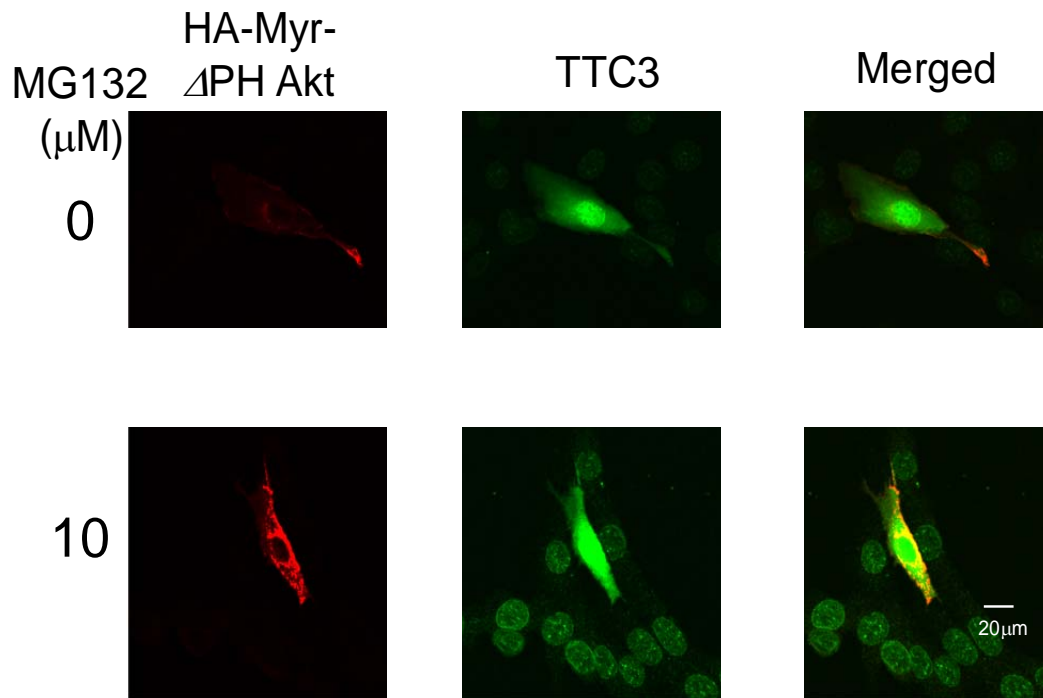
We examined whether MG132 can enhance the nuclear localization of phosphorylated Akt. MG132 treatment of NIH3T3 cells significantly enhanced nuclear co-localization of phosphorylated Akt (Ser473) with TTC3 in a dose escalation manner. Please note that MG132 also enhances TTC3 signals in the nucleus.

**Method:** NIH3T3 cells (ATCC) were cultured in DMEM in the presence of 10% FBS and Pen/Strep at 37°C with 5% CO<sub>2</sub>. The cells were treated with indicated concentration of MG132 (0~10 μM, Sigma) for 3.5 hours, fixed with 3.7% formaldehyde, stained with anti-TTC3 or phosphor Ser 473 Akt (#4051, Cell Signaling) and examined using a confocal microscope (Nikon).



**Supplemental data Fig. S8.**

***MG132 treatment enhanced co-localization of TTC3 with Myr-Akt***



NIH3T3 cells were transfected with Myr-Akt and treated with (lower panels) or without (upper panels) 10 $\mu$ M MG132. MG132 treatment of Myr-Akt transfected NIH3T3 cells clearly enhanced co-localization of TTC3 with Myr-Akt. The results were consistent with the finding that MG132 treatment significantly enhanced nuclear co-localization of phosphorylated Akt at Ser473 with TTC3 (both endogenous molecules) in NIH3T3 cells (Fig. S7).

**Method:** NIH3T3 cells were cultured in DMEM with 10% FCS with P/S, transfected with Myr-Akt, [HA-Myr- $\Delta$ PH (4-129) human-Akt1 in pECE vector (Kohn et al., 1996)] by PEI method as described, treated with 10  $\mu$ M MG132 (Sigma), fixed with 3.7% formaldehyde, stained with indicated antibodies anti-HA with anti-TTC3 and examined using a confocal microscopy (Nikon).

**Supplemental data Fig. S9. Supplemental data Fig. S9.**

*Ectopic re-introduction of TTC3 reverted the proliferative responses augmented by siRNA specific for TTC3.*

Panels, **A-D**.

Proliferation assays

were analyzed by

xCelligence (Real Time

Cell Analyzer, Roche)

using HT1080 cells

after transfected with

four different siRNAs

(designated as #1~#4,

see supplemental

information). Ectopic

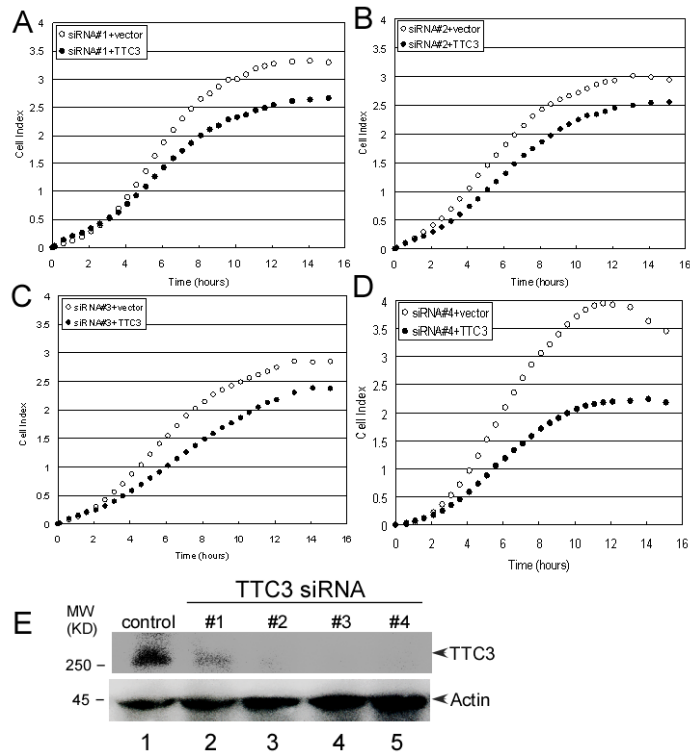
re-introduction of the

wild type TTC3 into HT1080 cells reverted the proliferative responses that were

enhanced by siRNAs specific for TTC3. Panel **E**. Suppression of the endogenous TTC3

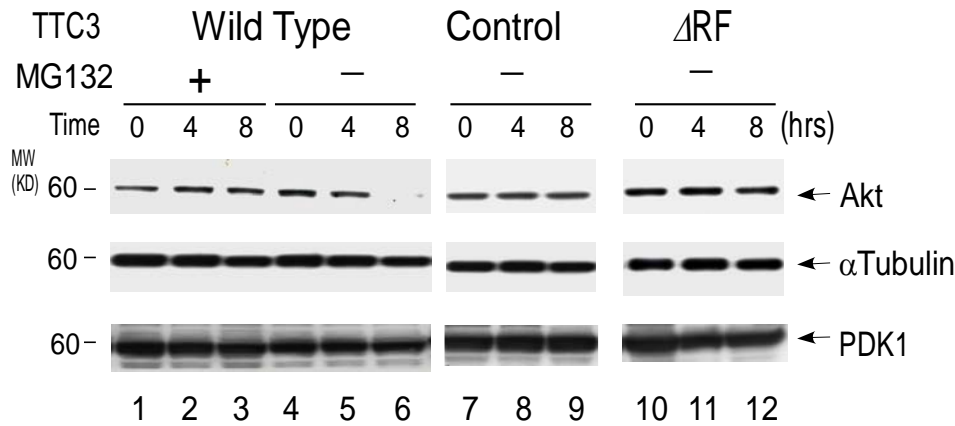
expression by each siRNA was shown by western blotting.

**Method:** Using HT1080 cells transfected with four different siRNA designated as #1~#4 siRNA specific for TTC3 (panels A~D, respectively, see supplemental information) at 40 nM concentration by CUY21 Pro-vitro (NEPA GENE Co. Ltd). 48 hours after the siRNA transfection, wild type TTC3 was re-introduced into the cells. Additional 24 hours later, the cells were re-seeded onto the E-plate 96 (Roche) and proliferative responses were analyzed by xCelligence (Real Time Cell Analyzer, Roche). For the western blotting of HT1080 cells to verify the suppression of the expression of endogenous TTC3, the cells were harvested and lysed using Brij97 lysis buffer (Laine et al., 2000), separated on SDS-PAGE, and immunoblotted using anti-TTC3 or anti-Actin antibodies.



**Supplemental data Fig. S10.**

***TTC3 induces proteasomal degradation of Akt in a specific manner.***



Wild-type TTC3, but not  $\Delta$ RF-TTC3-expressing cells (Amino acid 1-1957 of human TTC3, which lacks the RING-finger motif located in the C-terminal end of human TTC3, see Fig. 1A), efficiently induced proteasomal degradation of Akt in the presence of serum, which is known to activate Akt (top panel lanes 4-6 and 7-9, and 10-12, wild-type, control, and  $\Delta$ RF-TTC3 transfected cells, respectively), demonstrating that RF (Ring Finger motif) of TTC3 is required for efficient ubiquitination and proteasomal degradation of Akt, but not PDK1, consistent with the result that Akt could not interact with PDK1 in co-immunoprecipitation assays (Fig 1D). The observation supported the notion that TTC3 is a novel E3 ligase specific for Akt. 293T cells were cultured in DMEM supplemented with 10%FCS; under this condition, Akt is modestly phosphorylated and hence, susceptible for TTC3-dependent proteasomal degradation.

**Method :** 293T cells (ATCC) were cultured in DMEM in the presence of 10% FBS, transfected with indicated plasmids (WT-TTC3,  $\Delta$ RF-TTC3 in which Ring Finger domain of TTC3 was deleted, or vector control) by calcium phosphate method (Laine et al., 2000), 24 hours later, cells were treated with 10 $\mu$ M 17AAG (Basso et al., 2002) for 12 hours before 100 $\mu$ g/ml CHX treatment (Time 0) with or without 10 $\mu$ M MG132. Cells were harvested at indicated time points (0, 4, 8 hours after CHX treatment), lysed with Brijji 97 lysis buffer (Laine et al., 2000) containing 1mM Na<sub>3</sub>VO<sub>4</sub> and 10mM NaF with 10 $\mu$ M MG132, resolved onto SDS-PAGE, and immunoblotted (anti-Akt, anti- $\alpha$  tubulin, or anti-PDK1) using ECL.

## ***Detailed Experimental Procedures***

(antibodies and siRNAs utilized in the study were listed in the bottom)

### **Yeast Two-Hybrid screening**

Yeast two hybrid assays were essentially performed described previously (Laine et al., 2000). Y190 cells (Clontech) were transformed by the lithium acetate method with the "bait plasmid" (Akt2/PAS2-1) according to the manufacturer's protocol.  $\beta$ -gal positive clones were subsequently mated with Y187 yeast (MAT $\alpha$  gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 met2URA3::GAL-.lacZ) carrying pAS1-CYH2 without an insert or with SNF-1 or p53 to determine the specificity for the interaction. Two independent clones were identified that encode partial cDNA of human TTC3 (Tetratricopeptide repeat domain 3) [(Amino Acid from 890 to 1100) and (Amino Acid from 870 to 1102)].

### **Construction of TTC3 expression vectors**

Plasmid vector containing a partial cDNA fragment of human TTC3 was purchased from imaGenes (RZPD). The 5'-end of the cDNA of human TTC3 was extended by PCR amplification of using a pair of primers (HD173: 5'-ATGGACAATTTTGCTGAGGGAG and HD175: 5'-TATAGCCATC TTGAATTAAGCTTC) with cDNA pool derived from human Peripheral Blood Lymphocyte (PBL). The resulted PCR amplified full length TTC3 was subcloned into pCMV-Tag2 vector (Stratagene). Other forms of TTC3 were generated by the restriction digests or PCR amplifications. The nucleotide sequences in the final constructs used in the studies were confirmed.

### **Co-immunoprecipitation experiments (Fig. 1B, D, E, F, and 3D)**

Co-immunoprecipitation experiments were essentially performed as described previously (Laine et al., 2000). In brief, 293T cells (ATCC) were co-transfected with a total of 7.5  $\mu$ g of indicated plasmids per 10 cm dish. 72 hours after transfection, cells were washed twice with ice-cold PBS and lysed with ice-cold Brij 97 lysis buffer (see below) with proteinase inhibitors (Leupeptin, and AEBSF), and 10 $\mu$ M MG132. Lysates were precleared with protein G/protein A mixture (50% v/v, Pharmacia) for 1 hr,

immunoprecipitated with anti-HA or anti-Flag antibody (or other indicated antibodies) with mouse IgG as a control, run on SDS-PAGE (4-20% Tris glycine gel, Novax), and immunoblotted with indicated antibodies.

**Brij97 cell lysis buffer:**

0.875% Brij97 (Sigma), 0.125% NP40, 150mM NaCl, 10mM Tris HCl pH 7.5, and 2.5 mM EDTA with proteinase inhibitor mix (Leupeptin and AEBSF), phosphatase inhibitors (1 mM Na<sub>3</sub>VO<sub>4</sub> and 10 mM NaF) 10μM MG132(Sigma), and 5mM iodoacetamide were added where indicated. In cross-linking experiments using EGS (Ethylene glycobis sulfosuccinimidylsuccinate, Pierce) (Fig. 1C and Fig. 3A), Tris-HCL was replaced with phosphate buffered saline (PBS pH 7.4) as described (Laine et al., 2000).

**Co-immunoprecipitation assays using Hs52.Sk cells (Fig. 1C)**

Hs52.Sk cells [Down syndrome patient-derived skin fibroblast, ATCC, CRL-7031] were cultured in the presence of 10% FBS. The cells were washed twice with ice-cold PBS and treated with EGS (2mM, Ethylene glycobis sulfosuccinimidylsuccinate, Pierce) for 30 min., lysed with ice-cold Brij 97 lysis buffer in which Tris-HCL was replaced with phosphate buffered saline (PBS pH 7.4) and proteinase inhibitors (leupeptin and AEBSF), 10μM MG132, 5mM iodoacetamid, and phosphatase inhibitors (1 mM Na<sub>3</sub>VO<sub>4</sub> and 10 mM NaF), precleaned with protein G/protein A mixture (50% v/v, Pharmacia) for 1 hr, immunoprecipitated with isoform specific Akt antibodies (anti-Akt1 #07-416, anti-Akt2 #07-372, and anti-Akt3 #06-607, Upstate ) or control antibodies, resolved onto SDS-PAGE (8% Tris glycine gels), and immunoblotted with anti-TTC3 antibody. Expression of each Akt isofrom in whole cell lysate from Hs52.Sk cells was shown underneath by immunoblotting using isform specific antibodies (anti-Akt1 #07-558, anti-Akt2 #06-606, and anti-Akt3 #06-607, Upstate ).

**in vitro ubiquitination assays** (Fig. 2A and B)

An in vitro ubiquitination assay was performed as described (Hatakeyama et al., 2001; Lorick et al., 1999). In brief, reaction mixtures [1 μg recombinant TTC3 (see **Fig.**

S2), 0.1 µg recombinant rabbit E1 (Boston Biomedica), 1 µg recombinant human E2s, 0.5 U phosphocreatine kinase, 1 µg bovine ubiquitin (MBL)] in ubiquitin reaction buffer [25 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.2 mM ATP, 1 mM MgCl<sub>2</sub>, 0.3 mM dithiothreitol, and 1 mM creatine phosphate] were incubated for 2 hr at 30°C. The reaction was terminated by the addition of SDS sample buffer containing 4% β-mercaptoethanol and heating at 95°C for 5 min. Samples were subjected to immunoblotting with anti-ubiquitin antibody.

### **In Vitro Ubiquitination Assay (Fig. 2C)**

Recombinant TTC3 was generated by pFastBac HTC baculoviral system (Invitrogen) essentially described previously (Laine et al., 2000). The full length human TTC3 was subcloned into pFastBac HTC, and the resulted plasmid was co-transfected with Baculo-gold DNA into Sf-9 cells using Baculogold kit (PharMingen). Sf-9 cells were harvested after 60 hours, lysed with the following lysis buffer [50mM Tris-HCl buffer (pH 7.5), 1% Nonidet P-40, 10% glycerol, 137mM NaCl, 50mM β-glycerophosphate, 4µg/ml AEBSF, 4µg/ml aprotinin, 4µg/ml pepstatin A, and 4 µg/ml leupeptin], and spun at 15,000 x g for 30 min. The His x 6-tagged TTC3 was purified using a Ni<sup>2+</sup>-chelating affinity column after extensive washing using LiCl<sub>2</sub> washing buffer (lysis buffer plus 20mM imidazole and 0.5M LiCl) and eluted with elution buffer (lysis buffer plus 0.5M imidazole). Quality of recombinant TTC3 was verified by immunoblot. Active and inactive Akt (#14-276 active Akt1/PKBα and inactive Akt (#14-279 unactive Akt1/PKBα) were purchased from Upstate Biotech (USA). Ubiquitin was purchased from Sigma. Recombinant His<sub>6</sub>-UbcH4 and TTC were expressed and purified as described previously (Hatakeyama et al., 2001; Lorick et al., 1999). The *in vitro* ubiquitylation assay was performed essentially as described previously (Okumura et al., 2004) with some modifications. In brief, reaction mixtures (10µl) containing 30ng of His<sub>6</sub>-TTC, 0.1µg of recombinant human E1 (Biomol), 0.5 µg of purified His<sub>6</sub>-UbcH4, 3 µg of ubiquitin, 40 mM Tris-HCl (pH 7.4), 50mM KCl, 2mM ATP, 5mM MgCl<sub>2</sub>, 1mM dithiothreitol, 10% glycerol, and 25ng of unactive or active Akt were incubated for 1 h at 30°C. The reaction was terminated by adding SDS sample buffer containing 4% β-mercaptoethanol and heating at 95 °C for 5 min. Samples were subjected to

immunoblotting with anti-Akt antibody. Anti-HA-peroxidase (Roche) was utilized shown in the HA-blot, hence no immunoglobulin light chains were visible.

#### **Exogenous Akt2 ubiquitination** (Fig. 2D. Left panel)

Human 293T cell lines (ATCC) were transfected with pCMV6-HA-Akt2, wild type human TTC3 in pCMV-Tag2 vector (Stratagene, lane 4), and pCGN-HIS-Ub (HIS-tagged ubiquitin expression vector, lanes 2-4) by calcium phosphate method. Fifteen hours after the transfection, the cells were treated with 10 $\mu$ M MG132 (Sigma) for additional 6hrs, rinsed twice with ice cold PBS, and lysed with Brij97 lysis buffer in the presence of proteinase inhibitors (leupeptin and AEBSF), 10 $\mu$ M MG132, and 5mM iodoacetamid (Hatakeyama et al., 2001; Lorick et al., 1999). Immunoprecipitations were essentially performed as described (Laine et al., 2000). The resulted cell lysates were precleaned with 50% slurry of ProA/ProG beads mixture for 30 minutes, immunoprecipitated with anti-HA antibody (12CA5, Roche) and the samples were resolved onto SDS-PAGE (8% Tris-Glycine Gel), immunoblotted by anti-HA [middle panel, Anti-HA-Proxidase High Affinity (3F10) Roche] or Anti-His (top panel, #27-4710-01, Amersham), and detected by ECL. Anti-HA-peroxidase (Boehringer-Mannheim) was utilized shown in the HA-blot, hence no immunoglobulin light chains were visible. The expression of TTC3 was detected by anti-Flag [bottom panel, Anti-Flag (M2)-peroxidase (Sigma)].

#### **Endogenous Akt and AK1 ubiquitination** (Fig. 2D. Right panels)

Human 293T cell lines (ATCC) were transfected with pCGN-HA-Ub (HA-tagged ubiquitin expression vector) by calcium phosphate method. Fifteen hours after the transfection, the cells were treated with 10 $\mu$ M MG132 (Sigma) for additional 6hrs, rinsed twice with ice cold PBS, and lysed with Brij97 lysis buffer (Laine et al., 2000) in the presence of proteinase inhibitors (leupeptin and AEBSF), 10 $\mu$ M MG132, and 5mM iodoacetamid (Hatakeyama et al., 2001; Lorick et al., 1999).

The resulted cell lysates were precleaned with 50% slurry of ProA/ProG beads

mixture (Laine et al., 2000) for 30 minutes, immunoprecipitated with anti-Akt antibody (lane 7, #2966, Cell Signaling), anti-AK1 antibody (lane 6, #sc-28785, Santa Cruz), or control antibody (lane 5, sc-3877, Santa Cruz) and the samples were resolved onto 8% Tris-Glycine SDS Gel, immunoblotted by anti-HA [top panel, Anti-HA-Proxidase High Affinity (3F10), (Boehringer-Mannheim)], Anti-Akt (bottom panel, lane 7, # 9272, Cell Signaling), or Anti-AK1 (bottom panel, lane 6, #sc-28785, Santa Cruz) The expression of TTC3 was detected by anti-Flag [bottom panel, Anti-Flag (M2)-peroxidase (Sigma)]. and detected by ECL.

### **Endogenous Akt ubiquitination** (Fig. 2E)

Human 293T cell lines (ATCC) were transfected with indicated TTC3 mammalian expression constructs (wild type human TTC3 or  $\Delta$ RF-TTC3: Amino acid 1-1957 of human TTC3 that lacks C-terminal ring finger domain) in pCMV-Tag2 vector (Stratagene) along with pCGN-HA-Ub (HA-tagged ubiquitin expression vector). Fifteen hours after the transfection, the cells were treated with 10 $\mu$ M MG132 for additional 6hrs, rinsed twice with ice cold PBS, and lysed with Brij97 lysis buffer (Laine et al., 2000) with proteinase inhibitors (leupeptin and AEBSF) and 10 $\mu$ M MG132. The resulted cell lysates were precleaned with 50% slurry of ProA/ProG beads mixture for 30 minutes, immunoprecipitated with anti-Akt antibody (#9272, Cell Signaling), and the samples were resolved onto SDS-PAGE (4-20% Tris-Glycine Gel, Invitrogen), immunoblotted by anti-HA [left panel, Anti-HA-Proxidase High Affinity (3F10), Roche], or anti-Akt (right panel, # 9272, Cell Signaling), and detected by ECL. The expression of TTC3 was detected by anti-Flag [bottom panel, Anti-Flag (M2)-peroxidase (Sigma)].

### **K48R-Ubiquitination experiment** (Fig. 2F)

Human 293T cell lines (ATCC) were transfected with wild type, K48R pCGN-HA-Ub, (HA-tagged ubiquitin expression vector, in which lysine 48 was substituted into Arginine), or control vector by calcium phosphate method. Fifteen hours after the transfection, the cells were treated with 10 $\mu$ M MG132 for additional 6hrs, rinsed twice with ice cold PBS, and lysed with Brij97 lysis buffer (Laine et al., 2000) in

the presence of proteinase inhibitors (leupeptin and AEBSF), 10 $\mu$ M MG132, and 5mM iodoacetamid (Hatakeyama et al., 2001; Lorick et al., 1999).

The resulted cell lysates were precleaned with 50% slurry of ProA/ProG beads mixture (Laine et al., 2000) for 30 minutes, immunoprecipitated with anti-Akt antibody (#2966, Cell Signaling) and the samples were resolved onto 8% Tris-Glycine SDS Gel , immunoblotted by anti-HA [top panel, Anti-HA-Proxidase High Affinity (3F10), Roche] and detected by ECL. The expression of TTC3 was detected by anti-Flag [bottom panel, Anti-Flag (M2)-peroxidase (Sigma)].

#### **Akt ubiquitination by TTC3 siRNA**(Fig. 2G also see siRNA)

Mouse NIH3T3 cell lines (ATCC) were transfected with 64nM of small interfering RNAs, specific for mouse TTC3 (Invitrogen #HSS144355, HSS144356, and HSS144357) or control scrambled siRNA (Integrated DNA Technologies, Inc. IDT, USA) along with pCGN-HA-Ub (HA-tagged ubiquitin expression vector). Forty-eight hours after the transfection, the cells were treated with 10 $\mu$ M MG132 for additional 6hours, rinsed twice with ice cold PBS, and lysed with Brij97 lysis buffer (Laine et al., 2000)with proteinase inhibitors (Leupeptin and AEBSF), 10 $\mu$ M MG132, and 5mM iodoacetamide (Hatakeyama et al., 2001; Lorick et al., 1999). The resulted cell lysates were precleaned with 50% slurry of ProA/ProG beads mixture for 30 minutes, immunoprecipitated with anti-Akt antibody (#2966, Cell Signaling), and the samples were resolved onto SDS-PAGE (4-20% Tris-Glycine Gel, Invitrogen), immunoblotted by anti-HA [upper panel, Anti-HA-Proxidase High Affinity (3F10), Roche], or Anti-Akt (lower panel, # 9272, Cell Signaling), and detected by ECL. The expressions of endogenous TTC3 were shown by western blotting (top panel).

#### **siRNA experiments** (Fig. 2G, 3E, 6B)

In **Fig. 2G**, NIH3T3 cells (ATCC) were transfected with HA-Ubiquitin/pCGN and treated with 64 nM siRNA specific for mouse TTC3 (Invitrogen, #HSS144355, HSS144356, and HSS144357), immunoprecipitated with Akt and immunoblotted with anti-HA antibody to detect polyubiquitination of Akt (middle panel). In **Fig. 3E**, 293T cells ( ATCC) transfected with wild type TTC3 subsequently treated with indicated

concentrations (up to 64 nM concentration) of siRNA specific for human TTC3 or control scramble siRNA (Integrated DNA Technologies, Inc, IDT, supplemental information) by PEI. 72 hours later, the cells were harvested and immunoblotted by indicated antibodies. In **Fig.6B**, HT1080 cells (ATCC) were transfected with four different Small interfering RNAs (siRNA) targeting for human TTC3 or control scrambled RNA (Integrated DNA Technologies, Inc, IDT, supplemental information) using CUY21 Pro-vitro (NEPA GENE Co. Ltd). Twenty four hours after the transfection, the cells were re-seeded onto the E-plate (Roche) and the proliferative responses were measure red using xCelligence (Real-Time Cell Analyzer, Roche Applied Science) according to the manufacturer's protocol.

#### **Akt phosphorylation and efficiencies of ubiquitination** (Fig. 2H)

Human 293T cell lines (ATCC) were transfected with wild type HA-Akt2 or T308A-S473A-Akt2 (in which both Threonine 308 and Serine 473 were substituted into Alanine) along with wild type human TTC3 in pCMV-Tag2 vector (Stratagene) and pCGN-His-Ub (His-tagged ubiquitin expression vector) by calcium phosphate method. Fifteen hours after the transfection, the cells were treated with 10 $\mu$ M MG132 (Sigma) for additional 6hrs, rinsed twice with ice cold PBS, and harvested and lysed in Brij 97 lysis buffer with proteinase inhibitors (leupeptin and AEBSF), 10 $\mu$ M MG132, and 5mM iodoacetamid (Hatakeyama et al., 2001; Lorick et al., 1999). The resulted cell lysates were precleaned with 50% slurry of ProA/ProG beads mixture for 30 minutes, immunoprecipitated with anti-HA antibody (12CA5, Roche) and the samples were resolved onto SDS-PAGE (8% Tris-Glycine Gel), immunoblotted by anti-HA [bottom panel, anti-HA-Proxidase High Affinity (3F10) Roche] or anti-His (top panel, #27-4710-01, Amersham), and detected by ECL. The expression of TTC3 was detected by anti-Flag [bottom panel, Anti-Flag (M2)-peroxidase (Sigma)].

#### **Co-immunoprecipitation experiments of endogenous Akt with TTC3** (Fig.3A)

NIH3T3 cells (ATCC) were cultured in the presence (or absence) of 10% FBS to activate (or inactivate) Akt for 24 hours. The cells were washed twice with ice-cold PBS and treated with EGS (2mM, Ethylene glycobis sulfosuccinimidylsuccinate, Pierce) for

30 min., lysed with ice-cold Brij 97 lysis buffer in which Tris-HCl was replaced with phosphate buffered saline (PBS pH 7.4) and proteinase inhibitors (leupeptin and AEBSF), 10 $\mu$ M MG132, 5mM iodoacetamid, and phosphatase inhibitors (1 mM Na<sub>3</sub>VO<sub>4</sub> and 10 mM NaF), precleaned with protein G/protein A mixture (50% v/v, Pharmacia) for 1 hr, immunoprecipitated with anti-Akt (Cell signaling #2966) or control antibody (mouse IgG), resolved onto SDS-PAGE (4-20% Tris glycine gel, Novax), and immunoblotted. Levels of phosphorylated Akt, pan-Akt, or TTC3 were also shown by immunoblot.

### **GST pull-down assays of active/unactive form of Akt** (Fig. 3B)

Akt binding domain of human TTC3 corresponding to (Amino acid position 791-1101 of human TTC3) was subcloned into pGEX4T-2 (Pharmacia) by PCR amplification using a pair of primers (5'-ATCATCGTCGACCTTAAGAGAAA GTAATCCACCC and 5'-ATCATCGCGGCCGCTTACATGTCCCATAGCTT CTTATTG, note that Sall and NotI sites for subcloning purpose were underlined) using human TTC3 as a template. The resulted recombinant GST-TTC3 protein was generated according to the manufacture's protocol (Pharmacia). Reaction mixtures [60ng recombinant each Akt (active/unactive Akt, #14-276 active Akt1/PKB $\alpha$ , #14-279 unactive Akt1/PKB $\alpha$ , Upstate Biotech, USA) and 3 $\mu$ g GST-TTC3 or GST-only as a control] in Brij97 cell lysis buffer were incubated for 1 hr at 4 $^{\circ}$ C with gentle agitation. The resulted samples were washed three times and immunoblotted using anti pan-Akt antibody [Akt1/2(N-19), #sc1619 Santa Cruz, USA], phospho-Thr308:( #9275S Cell Signaling), or phospho-Ser473( #9271 Cell Signaling), and detected by ECL.

### **Cellular ubiquitination experiments** (Fig. 3C)

Human 293T cell lines (ATCC) were transfected with wild type TTC3 along with pCGN-HA-Ub (HA-tagged ubiquitin expression vector). Thirty six after the transfection, the cells were serum starved for overnight in the presence 10 $\mu$ M MG132. The cells were stimulated with 50nM PDGF (Peprotech) for 10 min., rinsed with ice-cold PBS, and lysed with Brij97 lysis buffer with protease inhibitors (leupeptin and AEBSF), phosphatase inhibitors (1 mM Na<sub>3</sub>VO<sub>4</sub> and 10 mM NaF), 10 $\mu$ M MG132, and 5mM

iodoacetamide (Hatakeyama et al., 2001; Laine et al., 2000; Lorick et al., 1999). The cell lysates were precleaned, immunoprecipitated with anti-Akt antibody. The samples were resolved onto SDS-PAGE, immunoblotted by indicated antibodies (anti-HA, anti-Akt, or anti-phospho-Thr308Akt), and detected by ECL.

### **Co-immunoprecipitation assay of wild type Akt and the phosphorylation defective Akt** (Fig. 3D)

Co-immunoprecipitation assays were performed described in (Laine et al., 2000). 293T cells (ATCC) were co-transfected with a total of 7.5 $\mu$ g of indicated plasmids (wild type Akt2, T308A-S473A-Akt2 with wild type TTC3) per 10 cm dish. 72 hours after transfection, cells were washed twice with ice-cold PBS and lysed with ice-cold Brij97 lysis buffer with proteinase inhibitors (Leupeptin and AEBSF). Lysates were precleaned with protein G/protein A mixture (50% v/v, Pharmacia) for 1 hr, immunoprecipitated with anti-HA or anti-Flag antibodies (or other indicated antibodies for verifying the expression of each construct) with mouse IgG as a control, ran on SDS-PAGE (4-20% Tris glycine gel, Novax), and immunoblotted with indicated antibodies.

### **siRNA experiments** (also see siRNA) (Fig.3E).

293T cells (ATCC) transfected with wild type TTC3 subsequently treated with indicated concentrations (up to 64 nM concentration) of siRNA specific for human TTC3 or control scramble siRNA (Integrated DNA Technologies, Inc, IDT, supplemental information) by PEI. 72 hours later, the cells were harvested and immunoblotted by indicated antibodies.

### **Myr-Akt induced Akt degradation by TTC3** (Fig. 3F and 4E)

293T cells were transfected with indicated plasmids [wild type TTC3, S378A-TTC3, or Myr-Akt [HA-Myr- $\Delta$ PH(4-129) human-Akt1 in pECE vector(Kohn et al., 1996)] by calcium phosphate. 24 hours later, cells were treated with 10 $\mu$ M 17AAG (Basso et al., 2002) for 12 hours before 100 $\mu$ g/ml CHX treatment (at time 0) with or without MG132 (Sigma). The cells were harvested at indicated time points, lysed with Brij 97 lysis buffer with 1mM Na<sub>3</sub>VO<sub>4</sub> and 10mM NaF, resolved onto SDS-PAGE, and

immunoblotted (anti-Akt, or anti-actin, or anti-HA) using ECL. Please note that Myr-Akt also exhibited time-dependent degradation (lanes 4-6, in second panel). Myr-Akt (Myr- $\Delta$ PH-Akt)(Kohn et al., 1996) migrated at approximately 50KD on SDS-PAGE gel, hence the amount of endogenous Akt migrated at 60 KD in size showed no increase in its expression levels in the presence of Myr-Akt (compare lanes 1 and 4 or lanes 7 and lane 10 in the top panels, in the absence and presence of Myr-Akt, respectively).

#### **Phosphorylation of S378 by Akt** (Fig. 4A)

293T cells were transfected with indicated plasmids (Flag-tagged Wild type TTC3, S378A-TTC3, or sham) by calcium phosphate method with (or without as indicated) Myr-Akt (constitutive active form of Akt, Upstate #21-151). 72 hours after transfection, cells were harvested, lysed with Brij 97 lysis buffer (with 1mM Na<sub>3</sub>VO<sub>4</sub> and 10mM NaF), immunoprecipitated with anti-Flag antibody, resolved onto SDS-PAGE, and immunoblotted [anti-Akt substrate (Cell Signaling #9611), anti-Flag (TTC3), or anti-phospho Ser473Akt to whole cell lysate] using ECL.

#### **in vitro Akt kinase assays of TTC3** (Fig. 4B)

TTC3 (wild type and S378A) constructs were generated by PCR amplification of a set of primer pairs (5'-ATCGAATTC-CAAGG TCGAAC AGC AAATAAG and 5'-CACTACTCGAGGT GAAAATTCAGG ATGACAG, both EcoRI and XhoI sites were underlined) and subcloned into EcoRI and XhoI sites of pGEX4T-2 vector (Pharmacia). In vitro kinase assays (IVK) were performed essentially using the Akt kinase assay kit (Cell Signaling) with the following modifications. TTC3-GST (wild type or S378A TTC3) fusion protein was generated according to manufacturer's protocol (Pharmacia), except using Brij97 lysis buffer. HA-Akt1 from 293T cells transfected with pCMV-HA-Akt1 was immobilized with anti-HA antibody (12CA5). IVK reaction was performed using TTC3-GST fusion protein (2.5 $\mu$ g) as substrates by incubating in the presence of 2 $\mu$ l (approximately 5ng) of immobilized Akt1 for the indicated 0-7 minutes at 30°C with gentle agitation. At indicated time points, the reactions were terminated by adding SDS sample buffer. The samples were separated on

SDS-PAGE and immunoblotted with phospho-(Ser/Thr) Akt substrate rabbit mAb (#9614, Cell Signaling) and detected by ECL.

#### **Akt specific siRNA experiment** (Fig. 4C)

Hs52.Sk cells (Down syndrome patient-derived skin fibroblast, ATCC, CRL-7031) were cultured in DMEM in the presence of 10% FCS and transfected with siRNA specific for Akt (#6211, Cell Signaling) using CUY21 Pro-vitro (NEPA GENE Co. Ltd). 72 hours later, the cells were lysed with Brij97 lysis buffer (Laine et al., 2000) with proteinase inhibitors (leupeptin and AEBSF), 10 $\mu$ M MG132, and 5mM iodoacetamide (Hatakeyama et al., 2001; Lorick et al., 1999), 1mM Na<sub>3</sub>VO<sub>4</sub> and 10mM NaF. Four microgram of the cell lysates were resolved onto SDS-PAGE, and immunoblotted with [anti-Akt (#9272, Cell Signaling), or anti-Actin, anti-phosphoAkt substrate (#9611, Cell signaling), or anti-TTC3] antibodies and detected using ECL.

#### **Endogenous Akt ubiquitination** (Fig. 4D)

Human 293T cell lines (ATCC) were transfected with indicated TTC3 mammalian expression constructs (wild type human TTC3,  $\Delta$ RF-TTC3: Amino acid 1-1957 of human TTC3 that lacks C-terminal ring finger domain, or S378A-TTC3) in pCMV-Tag2 vector (Stratagene) along with pCGN-HA-Ub (HA-tagged ubiquitin expression vector). Fifteen hours after the transfection, the cells were treated with 10 $\mu$ M MG132 and for additional 6hrs, rinsed twice with ice cold PBS, and lysed with Brij97 lysis buffer (Laine et al., 2000) with proteinase inhibitors (leupeptin and AEBSF), 10 $\mu$ M MG132, and 5mM iodoacetamide (Hatakeyama et al., 2001; Lorick et al., 1999). The resulted cell lysates were precleaned with 50% slurry of ProA/ProG beads mixture for 30 minutes, immunoprecipitated with anti-Akt antibody (#2966, Cell Signaling), and the samples were resolved onto SDS-PAGE (4-20% Tris-Glycine Gel, Invitrogen), immunoblotted by anti-HA [top panel, Anti-HA-Peroxidase High Affinity (3F10), Roche], or anti-Akt (middle panel, # 9272, Cell Signaling), or anti-Flag (bottom panel), and detected by ECL.

#### **Requirement of S378 for the degradation of Akt.** (Fig. 4E also see Fig. 3F)

293T cells were transfected with indicated plasmids [wild type TTC3 or

S378A-TTC3 together with Myr-Akt [HA-Myr- $\Delta$ PH(4-129) human-Akt1 in pECE vector(Kohn et al., 1996)] by calcium phosphate. 24 hours later, cells were treated with 10  $\mu$ M 17AAG (Basso et al., 2002) for 12 hours before 100 $\mu$ g/ml CHX treatment (at time 0) with or without MG132 (Sigma). The cells were harvested at indicated time points, lysed with Brij 97 lysis buffer with 1mM Na<sub>3</sub>VO<sub>4</sub> and 10mM NaF, resolved onto SDS-PAGE, and immunoblotted (anti-Akt, or anti-actin, or anti-HA) using ECL.

#### **Mitochondrial transmembrane potential (MTP) by rhodamine 123** (Fig. 4F)

293 T cells were transfected with a total 10 $\mu$ g of indicated plasmids DNA/10cm dish: 5 $\mu$ g of sham (vector control), wild type-TTC3, or S378A-TTC3 by calcium phosphate method. Forty eight hrs after transfection cells were stimulated with 50ng/ml of human TNF- $\alpha$  (PeproTech) for additional 6 hours at 37°C. Cells were stained with 2.5 $\mu$ M Rho123 (Molecular Probes) for 15 minutes with 2 $\mu$ g/ml propidium iodide (PI, Boehringer Mannheim). The Rho123 fluorescence intensity was analyzed in 1.5X10<sup>5</sup> cells using flow cytometry gating on PI negative cells (Laine et al., 2000).

#### **Luciferase Reporter Assays** (Fig. 4G)

Luciferase reporter assays were essentially performed described elsewhere (Hiromura et al., 2006) using Luciferase kit (Promega) using 293T cells transfected with indicated TTC3 vector (wild type, S378A, or control vector) along with luciferase NF $\kappa$ B reporter vector (Stratagene) by PEI (Polyscience Inc. #23966).

#### **Proliferation assay by Cell Titer AQueous One Solution Reagent** (Fig. 4H)

293T cells (ATCC) were transfected with 3 $\mu$ g of wild type TTC3 (or control vector or S378A-TTC3) in mammalian expression vectors by PEI method (Polyscience Inc. #23966). Two days after the transfection, the cells were seeded onto the 96 well plates (Corning #3596). Forty hours after the transfection, 20 $\mu$ l of the Cell Titer AQueous One Solution Reagent (Promega G3580) were added onto each well and 2 hours later, the absorption (OD 490nm) was measured by ELISA methods. The values shown were relative proliferative activities compared to the vector control out of 5 replicates. Statistical analysis was verified by student t test. Similar results were obtained in

another complete set of the independent experiment.

### **Subcellular fractionation and biochemical analysis of Akt ubiquitination and degradation** (Fig. 5A)

#### **Subcellular fractionation and biochemical analysis of Akt ubiquitination (Fig. 5A)**

293T cells were transfected with [(WT-TTC3 and HA-Ub in Fig. 5A) or (WT-TTC3, HA-Akt2 and His-Ub in Fig. S5)] plasmids by a calcium phosphate method. Fifteen hours after transfection, the cells were treated with 10 $\mu$ M MG132 for additional 6hrs before harvesting. Nuclear and cytoplasmic fractions were separated by hypotonic-hypertonic method described elsewhere. Briefly, harvested cells were washed with ice cold PBS, resuspended in Buffer A (10mM Hepes pH 7.9, 10mM KCl, 0.1mM EGTA, 0.1mM DTT supplemented with protease and phosphatase inhibitors), sit on ice for 15 min., add 10% NP-40, vortexed, spun, and the pellets were resolved by Buffer C (20mM Hepes pH 7.9, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT supplemented with protease and phosphatase inhibitors), vortexed for 15min. at 4 $^{\circ}$ C, and the nuclear fractions were collected by centrifuge. Akt or HA-Akt were immunoprecipitated from cytoplasmic and nuclear fraction using anti-Akt (#2966, Cell Signaling) or anti-HA (Roche) antibodies, resolved onto SDS-PAGE, and immunoblotted with indicated antibodies. The separation of the nuclear and cytoplasmic fractions was validated by western blotting using anti- $\alpha$ tubulin or anti-Lamin A/C.

#### **Akt degradation of nuclear and cytoplasmic fraction** (Fig. 5B-C)

293T cells were transfected with indicated TTC3 plasmids by calcium phosphate, 24 hours later, cells were treated with 10 $\mu$ M 17AAG (Basso et al., 2002) for 12 hours before 100 $\mu$ g/ml CHX treatment (time 0) with or without 10 $\mu$ M MG132. Cells were harvested at indicated time points, cytoplasmic and nuclear fractions were separated as described above, resolved onto SDS-PAGE, and immunoblotted using ECL. Quantitation of the gel was performed using NIH Image J and the relative intensities compared to the time 0 was presented (Fig. 5C).

#### **Co-localization experiment using a confocal microscopy** (Fig.5D-G and also see supplemental information of Fig. S6-S8)

NIH 3T3 cells (ATCC, Fig. 5D-F, S6-S8,) or Hs52.Sk cells (ATCC, Fig. 5G,) were cultured in DMEM with 10% FCS with P/S, fixed with 3.7% formaldehyde, stained

with indicated antibodies (anti-TTC3, phospho-Ser 473Akt, anti- $\alpha$  tubulin, or anti-Akt antibodies), or DAPI (4',6-diamidino-2-phenylindole, blue, Sigma), and examined using a confocal microscopy (Nikon). For the MG132 treatment, NIH3T3 cells were transfected without (Fig. S7) or with Myr-Akt (Fig. S8). The cells were then treated with indicated concentration of MG132 (0~10  $\mu$ M, Sigma) for 3.5 hours prior to harvest/fix the samples.

**Proliferation Assays using xCelligence (Roche)** (Fig. 6A, and also see siRNA of the supplemental information)

Proliferation assays were performed and analyzed by xCelligence (Real-Time Cell Analyzer, Roche Applied Science) using 293T cells transfected with indicated TTC3 constructs (Fig. 6A) treated with siRNA (at 64nM concentration, see supplemental information) or the control scrambled RNA (Integrated DNA Technologies, Inc, IDT, supplemental information) by PEI method (Polyscience Inc. #23966). 24 hours after the transfection, the cells were re-seeded onto the E-plate (Roche) and the proliferative responses were measure red using xCelligence (Real-Time Cell Analyzer, Roche Applied Science) according to the manufacturer's protocol.

**Proliferation Assays using xCelligence (Roche)** (Fig. 6B, and also see siRNA of the supplemental information)

Proliferation assays were performed and analyzed by xCelligence (Real-Time Cell Analyzer, Roche Applied Science) using HT1080 cells (Fig. 6B) treated with siRNA (at 40nM concentration, see details in the supplemental information) or 40nM concentration of the control scrambled RNA (Integrated DNA Technologies, Inc, IDT, supplemental information) by CUY21 Pro-vitro (NEPA GENE Co. Ltd). 24 hours after the transfection, the cells were re-seeded onto the E-plate (Roche) and the proliferative responses were analyzed using xCelligence (Real-Time Cell Analyzer, Roche Applied Science) according to the manufacturer's protocol.

### **Cell cycle analysis and western blot of DS cells** (Fig. 6C-6I)

**(Fig. 6C):** f3 (normal skin fibroblast, gift from Dr. Shimizu), HDF (normal human dermal fibroblasts, Cell Applications, Inc. #106-05a), or Hs52.Sk cells (Down syndrome patient-derived skin fibroblast, ATCC, CRL-7031) were cultured in DMEM and Jurkat cells (ATCC) or CMK85 cells [Down syndrome patient-derived megakaryoblastic leukemia cells (Sato et al., 1989)] were cultured in RPMI1640 both in the presence of 10% FBS and Pen/Strep at 37°C with 5% CO<sub>2</sub>. The harvested cells were washed with ice-cold PBS and lysed with Brij lysis buffer supplemented with protease (leupeptin and AEBSF), proteasome (10µM MG132) and phosphatase inhibitors (1mM Na<sub>3</sub>VO<sub>4</sub> and 10mM NaF) (Laine et al., 2000), analyzed onto SDS-PAGE gel and immunoblotted by indicated antibodies using ECL.

**(Fig. 6D):** CMK85 cells (Sato et al., 1989) were treated with indicated amount of siRNA specific for TTC3 and/or control siRNA as indicated (see supplemental information of siRNA) using CUY21 Pro-vitro (NEPA GENE Co. Ltd). The harvested cells were washed and lysed with Brij lysis buffer supplemented with protease (leupeptin and AEBSF), proteasome (10µM MG132) and phosphatase inhibitors (Laine et al., 2000), analyzed onto SDS-PAGE gel and immunoblotted by indicated antibodies.

**(Fig. 6E, 6F, and 6G):** 293T (ATCC), HDF (Human adult dermal fibroblasts, Cell Applications, Inc. #106-05a), Jurkat cells (ATCC), Hs52.Sk cells (Down syndrome patient-derived skin fibroblast, ATCC: CRL-7031), and CMK85 cells [Down syndrome patient-derived megakaryoblastic leukemia cells (Sato et al., 1989)] were used in this study. 293T cells (Fig. 6E), HDF cells (Fig. 6F), or Jurkat cells (Fig. 6G) were transfected with wild type TTC3/pCMV-Tag2A plasmid (or control) by PEI method (Fig. 6E, Polyscience Inc. #23966) or using CUY21 Pro-vitro (Fig. 6F and G, NEPA GENE Co. Ltd). The cells were harvested and fixed in 70% ethanol and keep at -20°C overnight. Fixed cells were twice washed in PBS and treated with 0.25U/ml RNase A in 3% FBS/PBS blocking buffer for 30min at room temperature. The resulted cells were washed in PBS and incubated with anti-Flag (M2) monoclonal antibody in blocking buffer for 30min at room temperature, which followed by staining with Alexa 488 labeled rabbit anti-mouse IgG. After washing the cells in PBS, the cells were stained

with 7AAD. The results were shown gated on the expression of Flag-TTC3 or control using CellQuest software ver. 3.3.

**(Fig. 6H):** 30nM siRNA specific TTC3 or control siRNA (see supplemental information) along with GFP-expression vector (pCMV-EGFP, 5 $\mu$ g per samples) to determine the transfection efficiencies were transfected into CMK85 cells using CUY21 Pro-vitro (NEPA GENE co. Ltd). The cells were cultured for 72 hours in RPMI 1640 containing 10% FBS and Pen/Strep, harvested, fixed with ethanol, stained with 7-AAD, and analyzed gated on the GFP-expressed cells using CellQuest software.

**(Fig 6I):** CMK85 cells transfected with HA-tagged Myr-Akt [HA-Myr- $\Delta$ PH(4-129) human-Akt1 in pECE vector(Kohn et al., 1996)], fixed with 70% ethanol, stained with anti-HA monoclonal antibody (12CA5) and Alexa 488 rebelled rabbit anti-mouse IgG together with 7-AAD. The cell cycle results were presented gated on anti-HA (Myr-Akt) expressed cells using CellQuest software.

#### **Antibodies used in this study:**

Polyclonal anti-TTC3 rabbit antibody was generated by immunizing rabbits using peptide CQGRDLLTEESPSGRG (AA 1996-2011 of human TTC3) (Asahi Techno Glass, Japan). Anti-HA peroxidase (3F10, #201389, Boehringer-Mannheim), anti-HA (12CA5, #1666606, Roche Diagnostics), anti-Flag (M2)-peroxidase (#A8592, Sigma), anti-Flag Affinity gel, (#2220, Sigma), anti-Akt (# 9272, or #2966, Cell Signaling), anti-Akt isoform specific antibodies (anti-Akt1 #07-416, anti-Akt2 #07-372, anti-Akt3 #06-607, anti-Akt1 #07-558, anti-Akt2 #06-606, and anti-Akt3 #06-607, Upstate), anti-phospho Ser473/Thr308 Akt (#4051 for Ser 473 and #9275 for Thr308, Cell Signaling), anti-p44/42 MAP (#9102, Cell Signaling), anti-phospho p44/42MAP (#9106, Cell Signaling), anti-FKHR (#9461, Cell Signaling), anti-phospho FKHR (#9462, Cell Signaling), anti-PDK1 antibody (#07-707, upstate), anti-lamin A/C (#612162, Transduction Laboratory), anti-Citron kinase (sc-1949, Santa Cruz), anti-AK1(#sc-28785, Santa Cruz), anti-His mAb (#27-4701-01, Amersham Bioscience), anti-actin (sc-1616, Santa Cruz), DAPI (4',6-diamidino-2-phenylindole #D9564, Sigma), mouse IgG<sub>1</sub> (#x0931, Dako Cytomation), and anti- $\alpha$ tubulin (DM1A, #9026, Sigma) were used.

**siRNA (Small interfering RNA):** Fig. 2G, 3C, 6B, 6D, Fig.S6A, and Fig S9)

Small interfering RNA targeting for human TTC3 (Fig. 3E, 6B, 6D, 6H, and S6A) were designed and purchased from Integrated DNA Technologies, Inc. (IDT, USA).

# 1. 5'-GGAGCACCAAGUAU UACAA GACCAA-3'

(Corresponding to the nucleotide position 4491-4515 of human TTC3)

# 2. 5'-GCAGUAUGCUGACAAGAUUAAAUCC-3'

(Corresponding to the nucleotide position 2675-2499 of human TTC3)

# 3. 5'-GAAGGAGAGUGAAGUGUAUAAGCTA-3'

(Corresponding to the nucleotide position 4944-4968 of human TTC3)

# 4. 5'-AGAGGAUUAUGAAGAGAGUCAUCAG-3'

(Corresponding to the nucleotide position 4881-4905 of human TTC3)

Small interfering RNAs, specific for mouse TTC3 (Fig. 2G) were purchased from Invitrogen (#HSS144355, HSS144356, and HSS144357). Control scramble RNA (DS Scrambled Neg Control Duplex) were purchased from Integrated DNA Technologies, Inc. (IDT, USA).

### Supplemental References

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