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Essential role of PH domain and leucine-rich repeat protein phosphatase 2 in Nrf2 suppression via modulation of Akt/GSK3β/Fyn kinase axis during oxidative hepatocellular toxicity

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Instances of sustained oxidative activity have been shown to involve dysregulation of Nrf2-mediated transcriptional induction; however, mechanisms warranting Nrf2-repression remain unclear. In this study, using primary rat hepatocytes, we have attempted to identify factors that may negatively influence Nrf2 survival pathway. Though studies indicate a conspicuous association between Akt and Nrf2, a confirmatory link between the two is unaddressed. On inhibiting PI3K/Akt pathway, we observed compromised activities of antioxidant and detoxification enzymes culminating in oxidative cytotoxicity. This was accompanied by reduced nuclear retention of Nrf2 and its ARE binding affinity, increased Nrf2 ubiquitination and concurrent decline in its downstream targets. Moreover, Akt inhibition enhanced nuclear translocation as well as phosphorylation of Fyn kinase, an enzyme linked to Nrf2 degradation, by relieving GSK3β from phosphorylation-mediated repression. The involvement of Akt and Fyn kinase in influencing Nrf2 signaling was further confirmed in oxidatively stressed hepatocytes by using *tert*-butyl hydroperoxide (tBHP). tBHP-induced decrease in Nrf2 levels was associated with enhanced Fyn kinase phosphorylation, Fyn kinase nuclear translocation and decreased levels of phosphorylated GSK3β(Ser9) in a time-dependent manner. Interestingly, tBHP induced site-specific deactivation of Akt as only Akt(Ser473) phosphorylation was observed to be affected. Further, protein expression as well as nuclear localization of PHLPP2, a phosphatase specific for Akt(Ser473), was found to be significantly enhanced in tBHP-stressed hepatocytes. Silencing of PHLPP2 not only resulted in considerable restoration of Nrf2 signaling, enhanced Nrf2-ARE binding and reduced Nrf2 ubiquitination but also significantly suppressed tBHP-induced ROS generation and alterations in mitochondrial permeability. We infer that cellular PHLPP2 levels may aggravate oxidative toxicity by suppressing Nrf2/ARE transcriptional regulation via Akt(Se473)/GSK3β/Fyn kinase axis. The study indicates that PHLPP2 could serve as a new target for developing strategies to manage pathological conditions exacerbated due to oxidative stress.
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Exposure to a large number of potentially toxic compounds renders the liver, in particular, susceptible to injury. Xenobiotic-induced hepatocellular damage is a much studied and clinically relevant phenomenon. The toxicity of most xenobiotics is associated with their biotransformation or metabolism that is frequently coupled with irregularities in cellular oxidant/antioxidant balance.[1] As impairment of hepatocellular functions may be a prelude to hepatic failure, an understanding of the mechanisms by which toxic compounds inflict irreversible damage to cells is crucial for alleviation of liver injury.

Nrf2 (nuclear factor erythroid 2-related factor 2), a redox sensitive transcription factor, coordinates the controlled expression of antioxidant genes so as to reinstate redox homeostasis in an event of oxidative prevalence.

Nonetheless, studies indicate that a number of pathological circumstances involving oxidative imbalances are correlated with perturbed activity or stability of Nrf2 itself.[2–6] Considerable research has been conducted to delineate the mechanisms responsible for regulating Nrf2 responses within the cell. The phosphatidylinositol 3′-kinase (PI3K)/Akt pathway forms an important component of cell survival,[7] which is activated in response to oxidative stress.[8] Previous studies have reported functional interactions between the PI3K/Akt pathway and Nrf2 activation,[9–12] but no direct relationship has yet been confirmed. Fyn kinase, a member of Src family of tyrosine kinases, is believed to influence stability and nuclear accumulation of Nrf2 by promoting its degradation.[13] Fyn kinase has been demonstrated to localize to the nucleus

during stress and shares an inverse relationship with nuclear Nrf2 density.[14,15]

The PHLPP isoforms (PH domain and leucine-rich repeat protein phosphatases) regulate phosphorylation of Akt kinases at Ser473 residue. Recently, PHLPP knockout has been reported to protect the brain against ischemic insult.[16] Studies indicate that the cellular levels of PHLPP2, the isoform that specifically targets Akt1,[17] affect proliferative potential of tumorigenic cells,[18–20] highlighting its role in promoting apoptotic events. However, the importance of PHLPP2 in regulation of Akt activity and its subsequent effect on cell survival mechanisms has not yet been addressed in relation to Nrf2 responses in oxidatively stressed cells.

Targeting the regulatory processes that act in the development of oxidative stress-induced toxicity may be a proper strategy to restore a disturbed balance. Thus, a clear understanding of the molecular events that influence the cell's potential to thwart damaging effects of reactive oxygen species (ROS) is indispensible. In this study, we have explored the novel relationship shared between PHLPP2, Akt1 and Fyn kinase in determining Nrf2 stability and hence its activity during oxidative stress-evoked hepatocellular toxicity. The study suggests that inhibition of PHLPP2 could be a promising approach to reinstate cell defense mechanism, which has been compromised owing to dysregulation in Nrf2 signaling during excessive ROS generation.

Results

Inhibition of Akt diminishes cellular antioxidant defense triggering oxidative stress-mediated death of hepatocytes. To validate the involvement of Akt in regulating redox balance through Nrf2 signaling mechanism, we measured the activities of some of the key antioxidant and detoxification enzymes that are downstream Nrf2 targets in primary rat hepatocytes exposed to varying concentrations of LY294002, a PI3K inhibitor. As expected, inhibition of Akt activation led to significant decline in the activities of key antioxidant (thioredoxin reductase (TrxRed), glutathione reductase (GR), glutathione peroxidase (GPx)) and detoxification enzymes (glutathione *S*-transferase (GST) and NAD(P)H quinone oxidoreductase 1 (NQO1)) in a concentration-dependent manner (Figure 1a). LY294002 treatment also diminished the nuclear as well as cytoplasmic levels of reduced glutathione (GSH), indicating the important role of Akt in maintaining balanced redox environment in sub-cellular compartments (Figure 1b). Henceforth, the disturbed defensive mechanism of the cell warranted enhanced oxidative stress as indicated by estimation of DCF fluorescence and Ethidium/DHE ratio (Figures 1c and d; Supplementary Figure S1a). Increased cellular oxidative burden not only perturbed the mitochondrial membrane potential (Figure 1e) but also compromised the viability of hepatocytes, which was observed to decrease significantly with increasing LY294002 concentration (Supplementary Figure S1b). In accordance with the role of Akt in cell survival pathway, the data suggest that inhibition of Akt undermines antioxidant defense mechanisms, which thus exacerbate free radical accumulation and related functional insufficiencies culminating in cell toxicity.

Inhibition of Fyn kinase elevates cellular antioxidant defense against intra-cellular free-radical generation. Fyn kinase has been reported to destabilize Nrf2,[13] which suggests its inverse relevance to cellular oxidative burden. The activities of redox enzymes (TrxRed, GR, GPx, GST and NQO1) were observed to increase with increasing concentration of (4-amino-5-(methylphenyl)-7-(t-butyl)pyrazolo-(3, 4-d)pyrimidine (PP1), inhibitor of Fyn kinase activation, reaching their maximum at 15 μM concentration as compared with control (Figure 2a). Thereafter, a consistent drop in the activity of the studied enzymes was observed, reaching values comparable to that of control at 25 μM PP1 concentration. Moreover, PP1 exposure also enhanced the nuclear as well as overall levels of GSH (Figure 2b). Accordingly, treatment with PP1 exhibited considerable decrease in endogenous ROS and superoxide levels as compared with control (Figures 2c and d; Supplementary Figure S2a). Inhibiting Fyn kinase did not indicate any significant loss of cell viability (Supplementary Figure S2b) and also did not appear to modulate mitochondrial membrane polarity (Figure 2e). Taken together, the data confirms that intervention in Fyn kinase activation augments cell's resistance to free radical generation and/or accumulation.

Akt regulates Nrf2 signaling mechanism by repressing Fyn kinase phosphorylation and its nuclear localization. As the antioxidant and detoxification enzymes are under the direct regulation of Nrf2, we further evaluated the level of Nrf2 protein expressed in the hepatocytes upon inactivation of Akt and Fyn kinase enzymes. Significant decline in Nrf2 and its target proteins' levels first appeared around 30 μM concentration of LY294002 as indicated in Figure 3a (left panel). On the other hand, a consistent increase in the levels of Nrf2, NQO1 and HO1 (heme oxygenase 1) protein levels could be observed as the concentration of PP1 (Fyn kinase inhibitor) increased. In addition, inhibiting Akt pathway decreased the levels of Nrf2 within the nuclear compartment while Fyn kinase inhibition followed the opposite trend (Figure 3b). The immunofluorescent detection of sub-cellular Nrf2 localization further supported the above conclusion that Fyn kinase inactivation promotes nuclear retention of Nrf2 (Figure 3e). Not only this, intervention of the Akt and Fyn kinase pathway also affected the functional activity of Nrf2. LY294002 at 30 μM caused upto 40% decrease in antioxidant redox element (ARE)-binding affinity of Nrf2, while Fyn kinase inhibition with 15 μM PP1 exhibited upto 38% increase in its functional capacity (Figure 3c). The results clearly indicate the opposing roles of Akt and Fyn kinase in terms of mechanistic regulation imposed on Nrf2 signaling.

We observed that at 30 μM LY294002 concentration reduction in phosphorylated levels of both Akt Thr308 and Ser473 residues was accompanied by a significant decrease in GSK3β(Ser9) phosphorylation (Figure 3a). GSK3β is the immediate downstream effector molecule of Akt that is deactivated when phosphorylated by Akt at its Ser9 residue. Studies have also proven that GSK3β is the upstream activator of Fyn kinase phosphorylation.[21] Thus, accordingly, we also observed a significant increase in phosphorylation
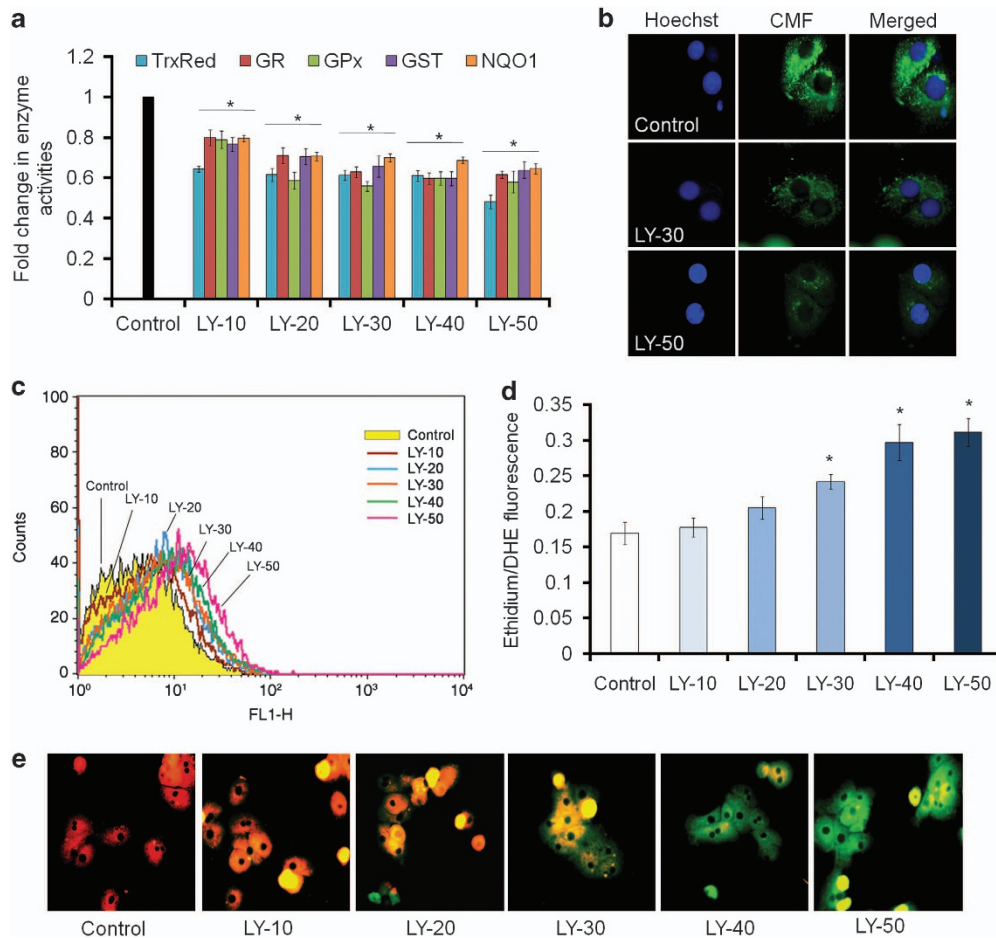
Figure 1 Akt inhibition induces oxidative stress due to perturbed antioxidant balance. Hepatocytes were treated with varying concentrations of LY294002 (10–50 μM) for 30 min. (**a**) Alteration in enzyme activities of TrxRed, GR, GPx, GST and NQO1 was assessed in LY294002-stressed hepatocytes. (**b**) Sub-cellular GSH levels assessed using fluorescence microscopy of CMFDA-stained hepatocytes treated with 30 μM and 50 μM LY294002 for 30 min; (magnification $\times$ 63). ROS generation was assessed by (**c**) FACS analysis of DCF-stained cells and (**d**) fluorimetric estimation of Ethidium/DHE fluorescence ratio. (**e**) Alteration of mitochondrial membrane potential assessed by JC-1 staining of LY294002-treated hepatocytes (magnification $\times$ 40). The micrographs represent images obtained after merging of red and green fluorescence channels. The data are presented as mean $\pm$ S.E. of at least three independent experiments. *$P < 0.05$ compared with control

status of Fyn kinase upon LY294002 exposure. Phosphorylation of Fyn kinase has been found to be associated with its nuclear localization. Western blotting analysis (Figure 3b) together with immunofluorescent imaging (Figure 3f) confirmed that inactivation of Akt pathway evokes activation and nuclear localization of Fyn kinase. Inhibition of Fyn kinase using PP1 did not result in any change in phosphorylation status of both Akt Ser473 residue and GSK3β(Ser9), suggesting that Fyn kinase functions downstream of Akt pathway. However, significant reduction in phosphorylation of Akt at Thr308 residue could be observed (Figure 3a). This might be indicative of feedback regulation imposed by Fyn kinase on Thr308 site of Akt. Further, in accordance with the role of Fyn kinase in promoting Nrf2 degradation, treatment with PP1 subsided the levels of ubiquitinated Nrf2 as compared with control (Figure 3d, right panel), but LY294002 exposure promoted Nrf2 ubiquitination (Figure 3d, left panel). The data collectively demonstrate that PI3K/Akt pathway imposes its regulation on Nrf2 signaling by checking Fyn kinase activation.

***Tert*-butyl hydroperoxide (tbhp)-induced increase in PHLPP2 (PH-domain and leucine-rich repeat protein phosphatase 2) causes site-specific Akt deactivation resulting in impairment of Nrf2 signaling.** Nrf2 is a key cellular transcription factor regulating the expression of proteins involved in the maintenance of redox homeostasis. Reports suggest that toxicity arising due to oxidative damage is a result of impairment of redox balance. In order to ascertain whether an event of oxidative toxicity implies any dysregulation in Nrf2 signaling due to intervention of pathway relating Akt and Fyn kinase, we treated primary hepatocytes with tBHP, a commonly used oxidative stress inducer. We observed that a concentration of 250 μM tBHP was sufficient to elicit significant cell death of hepatocytes (Supplementary Figure S3), which corresponded to increased free radical generation and loss of mitochondrial membrane potential (data not shown). Western blotting analysis demonstrated that tBHP exposure significantly decreased total Nrf2 levels at 120 and 180 min (0.7- and 0.8-fold, respectively), but significant reduction in its target
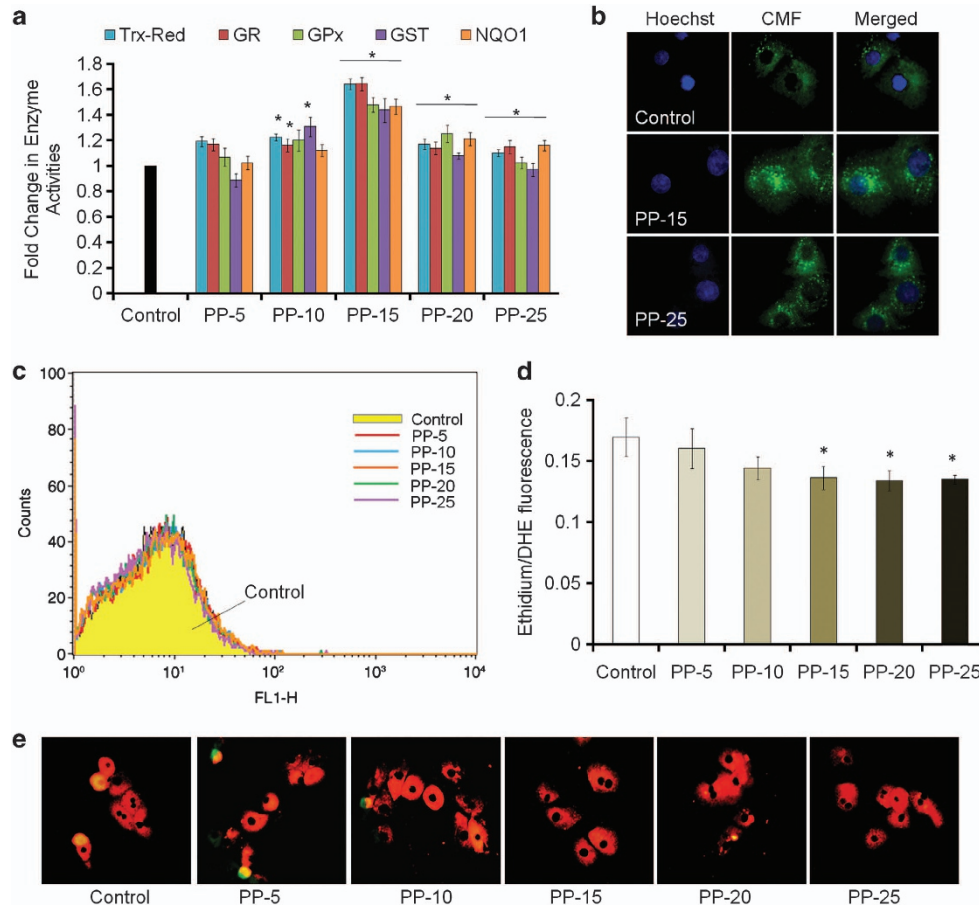
Figure 2 Fyn kinase inhibition subdues endogenous oxidative load and enhances cellular antioxidant defense. Hepatocytes were treated with varying concentrations of PP1 (5–25 μM) for 30 min. (**a**) Alteration in enzyme activities of TrxRed, GR, GPx, GST and NQO1 in PP1-stressed hepatocytes. (**b**) Sub-cellular GSH levels assessed using fluorescence microscopy of CMFDA-stained hepatocytes treated with 15 μM and 25 μM PP1 for 30 min; (magnification $\times$ 63). ROS generation was assessed by (**c**) FACS analysis of DCF stained cells and (**d**) fluorimetric estimation of Ethidium/DHE fluorecence ratio. (**e**) Alteration of mitochondrial membrane potential assessed by JC-1 staining of PP1-treated hepatocytes (magnification $\times$ 40). The micrographs represent images obtained after merging of red and green fluorescence channels. The data are presented as mean $\pm$ S.E. of at least three independent experiments. *$P < 0.05$ compared with control

proteins HO1 and NQO1 became apparent as early as 60 min (Figure 4a). This could be explained by the reason that nuclear retention of Nrf2 started to diminish from 60 min time period of tBHP exposure (Figure 4d). Western blotting analysis of key components of Akt signaling pathway revealed that tBHP stress did not affect the total Akt1 levels as well as phosphorylation of Akt at Thr308 residue (except for the initial 1.5-fold increase at 15-min exposure period, Figures 4a and b); however, consistent time-dependent reduction with respect to phosphorylation of Akt at Ser473 residue could be observed (Figures 4a and b). Accordingly, PDK1, which is responsible for phosphorylating Akt at its Thr308 residue, showed no change with respect to its phosphorylation. Further, while phosphorylation of PTEN(-Ser380) decreased (which implies enhanced PTEN activity), a remarkable decline in GSK3β phosphorylation was detected. As earlier reports and our data here (Figure 3) confirm that Fyn kinase is associated with suppression of Nrf2 activity, we assessed the levels of phosphorylated Fyn kinase as well as its nuclear density. tBHP treatment led to significant enhancement in levels of phosphorylated Fyn kinase (Figures 4a and b), with concomitant increase in its

nuclear localization in tBHP-treated hepatocytes (Figures 4d and e). This suggests that oxidative toxicity of tBHP is due to subdued Nrf2 functionality brought about by lifting the repression imposed by Akt(Ser473)-GSK3β(Ser9) wing on Fyn kinase activation.

As our data revealed specific downmodulation of Akt phosphorylation at Ser473 residue, we hypothesized that either decreased phosphorylation or enhanced selective dephosphorylation of Ser473 residue may be involved. To testify the two possibilities, we checked the levels of phosphorylated mTORC2 (kinase) as well as PHLPP2 (phosphatase), both of which specifically target Ser473 residue of Akt1.[17,22] Though no significant alteration in levels of phospho-mTORC2 were revealed except for a 0.7-fold dip at 180 min, we did observe pronounced increase in levels of PHLPP2 as early as 30 min time period of incubation with 250 μM tBHP (Figure 4c and Supplementary Figure S4a). This highlights the involvement of phosphatase PHLPP2 in downregulating Akt activity. Apart from this, we also observed increased nuclear levels of PHLPP2 that corresponded to decreasing phosphorylation of Akt Ser473 within the nucleus (Figure 4d). Immunofluorescent detection also
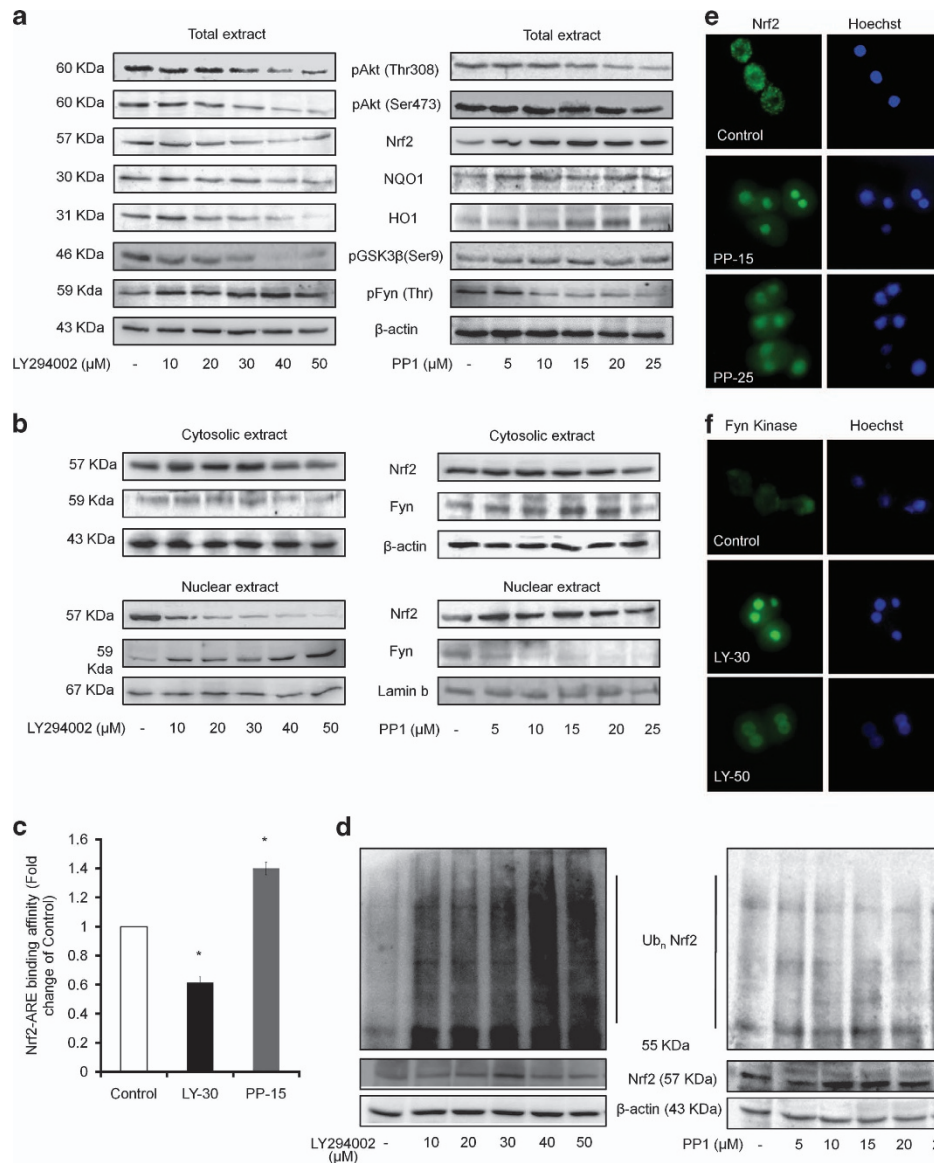
Figure 3 Inhibition of Akt suppresses Nrf2-regulated survival pathway by enhancing Fyn kinase activation and its nuclear translocation. LY294002 (10–50 μM) and PP1 (5-25 μM) were used to inhibit Akt and Fyn kinase, respectively. Western blotting analysis of (**a**) Nrf2 and its downstream targets NQO1 and HO1 and phosphorylated forms of Akt, GSK and Fyn kinase in total cellular extract and (**b**) Nrf2 and Fyn kinase in nuclear and cytosolic extracts. β-Actin was used as an endogenous control for total and cytosolic extracts while lamin b was used as a reference protein for nuclear extract. (**c**) Estimation of enzyme-linked immunosorbent assay-based Nrf2-ARE binding affinity in nuclear lysates obtained from hepatocytes treated with 30 μM LY294002 and 15 μM PP1 for 30 min. (**d**) Levels of ubiquitinated Nrf2 were analyzed by immunoprecipitating Nrf2 protein followed by western blotting detection with anti-ubiquitin antibody. (**e**) Nuclear translocation of Nrf2 upon Fyn kinase inhibition was assessed by immunofluorescence staining of hepatocytes for Nrf2 (green) and Hoechst (blue); (magnification $\times$ 40). (**f**) Nuclear translocation of Fyn kinase upon Akt inhibition was assessed by immunofluorescence staining of hepatocytes for Fyn kinase (green) and Hoechst (blue); (magnification $\times$ 40). The data are presented as mean $\pm$ S.E. of at least three independent experiments. $*P < 0.05$ compared with control

supported the increased sub-cellular levels of PHLPP2 (Supplementary Figure S4b). The findings clearly demonstrate the involvement of PHLPP2 in influencing Nrf2-regulated survival pathway through site-selective Akt deactivation.

PHLPP2 induction imposes negative regulation on Nrf2 signaling during tBHP-induced hepatocellular toxicity.
We reasoned that if PHLPP2 is responsible for blocking Nrf2 activation via modulation of Akt(Ser473)–GSK3β–Fyn kinase axis, then knockdown of PHLPP2 should restore this activation. A 15-nM concentration of small interfering RNA

(siRNA) targeting PHLPP2, which produced nearly 60–70% knockdown after 48 h incubation (Supplementary Figure S5b), was used for silencing experiments. Stimulations with 250 μM tBHP (for 90 min) followed 48 h after transfection. Our data reveal that PHLPP2-silencing not only significantly enhanced phosphorylation of Akt(Ser473) and GSK3β(Ser9) in tBHP-treated hepatocytes but also suppressed Fyn kinase activation, as indicated by decline in Fyn kinase phosphorylation (Figures 5a and b). Knockdown of PHLPP2 also led to a substantial increase in nuclear phospho-Akt(Ser473), which was accompanied by decreased nuclear retention of
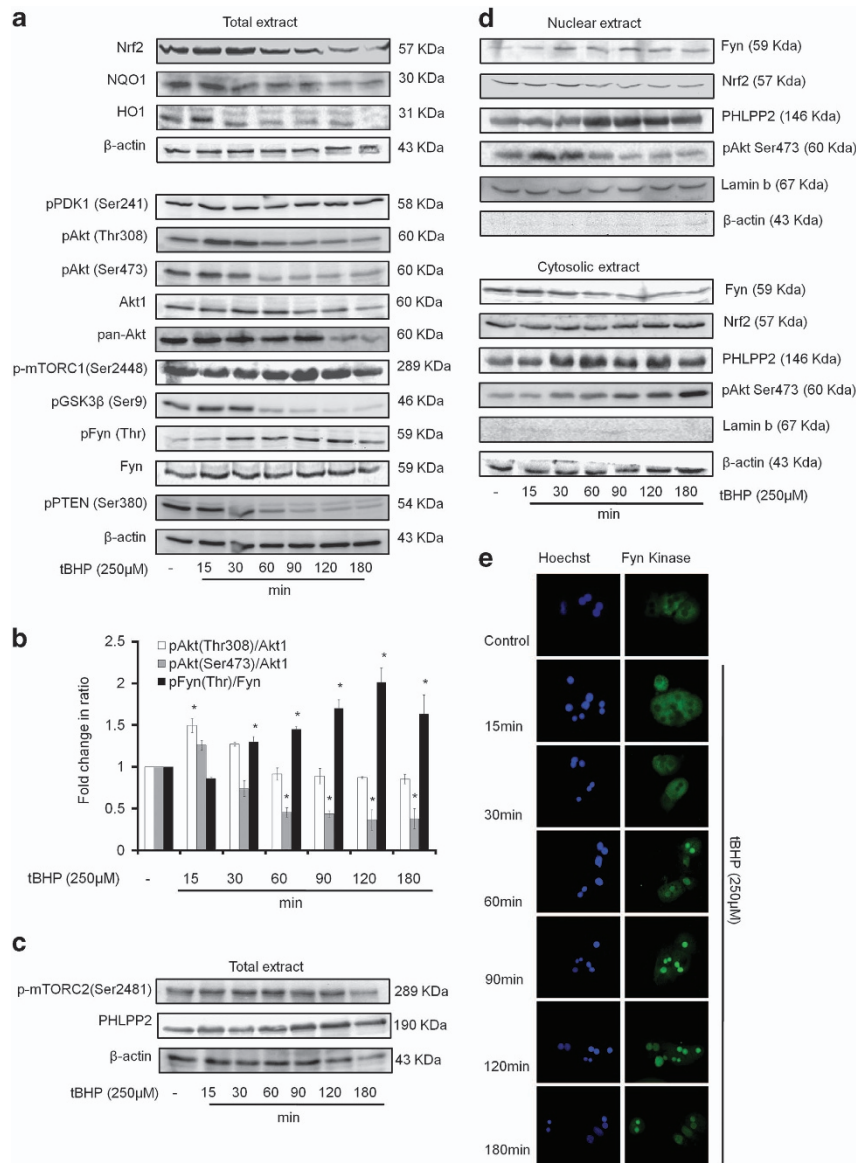
Figure 4 tBHP-induced PHLPP2 protein expression mediates site-specific Akt deactivation leading to Fyn kinase nuclear translocation and compromised Nrf2 signaling. Hepatocytes were treated with 250 μM tBHP for different time periods (15–180 min). (a) Immunoblot detection of key proteins involved in Nrf2 and Akt pathway. β-Actin was used as endogenous control to normalize the protein expression values. (b) Graph representing change in ratio of phosphorylated/total Akt and Fyn kinase during exposure to tBHP. Western blotting images of (c) PHLPP2 and mTORC2 in total cellular extract and (d) Nrf2, Fyn kinase, PHLPP2 and Akt(Ser473) in nuclear and cytosolic extracts. β-Actin lamin b were used as reference controls for cytosolic and nuclear extracts. (e) Immunofluorescence staining of hepatocytes for Fyn kinase (green) and Hoechst (blue) illustrating nuclear translocation of Fyn kinase upon tBHP exposure; (magnification $\times$ 40). The data are presented as mean $\pm$ S.E. of at least three independent experiments. *$P < 0.05$ compared with control

Fyn kinase (Figure 5c). Consequently, blocking PHLPP2 expression restored Nrf2 activation as indicated by enhanced NQO1, HO1 levels (Figure 5a), increased nuclear retention of Nrf2 (Figures 5c and d), increased Nrf2 stability (Figure 5e) and Nrf2-ARE-binding affinity (Figure 5f) as compared with tBHP-treated normal hepatocytes. In all, the data confirm that PHLPP2 imposes negative regulation on Nrf2 survival mechanism through suppression of Akt-induced Fyn kinase deactivation.

PHLPP2 knockdown checks tBHP-induced oxidative stress. As we speculated that during tBHP exposure hindered Nrf2 responses result in oxidative overload leading to hepatocellular death, PHLPP2 knockdown should therefore prevent tBHP-mediated free radical generation through potentiation of Nrf2 signaling. Conforming to the positive outcome of PHLPP2-silencing on Nrf2 activation, we observed a significant reduction in ROS and superoxide generation (Figure 6a) as well as mitochondrial depolarization (Figure 6b) induced due to tBHP exposure. In addition, considerable enhancement in sub-cellular GSH levels could also be observed (Figure 6c) by blocking PHLPP2 expression. The data collectively manifest plausible relationship between PHLPP2 and Nrf2-regulated redox homeostasis (Figure 7) and the ensuing cell survival/death mechanism.
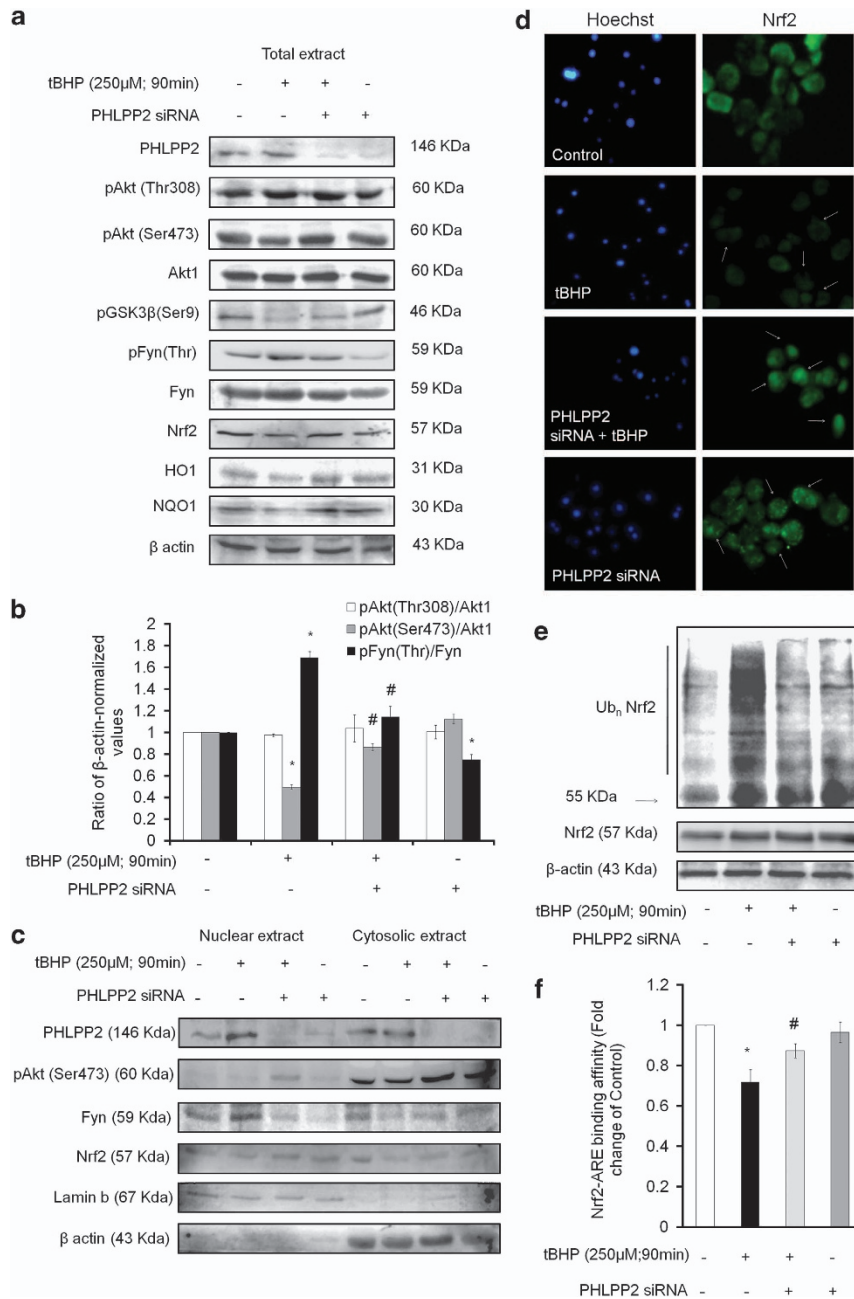
Figure 5 PHLPP2-silencing restores Nrf2 signaling by promoting Akt-induced Fyn kinase deactivation during tBHP exposure. Normal and PHLPP2-silenced hepatocytes were challenged with 250 μM tBHP for 90 min. (**a**) Shows immunoblot detection of key proteins involved in Nrf2 and Akt pathway. (**b**) Graph representing change in ratio of phosphorylated/total Akt and Fyn kinase in normal and PHLPP2-silenced hepatocytes treated with tBHP. (**c**) Western blotting images of PHLPP2, pAkt(Ser473), Nrf2 and Fyn kinase in nuclear and cytosolic extracts. β-Actin was used as an endogenous control to normalize protein expression in cytosolic extracts while lamin b was used as a reference protein for nuclear extract. (**d**) Immunofluorescence staining of hepatocytes for Nrf2 (green) and Hoechst (blue) illustrating nuclear translocation of Nrf2 (magnification $\times$ 40). (**e**) Levels of ubiquitinated Nrf2 were analyzed by immunoprecipitating Nrf2 protein followed by western blotting detection with anti-ubiquitin antibody. (**f**) Estimation of enzyme-linked immunosorbent assay-based Nrf2-ARE binding affinity in nuclear lysates obtained from normal and PHLPP2-silenced hepatocytes treated with tBHP. The data are presented as mean $\pm$ S.E. of at least three independent experiments. $^*P < 0.05$ compared with control; $^\#P < 0.05$ compared with tBHP

Discussion

The normal balance between activated oxygen generation and cellular antioxidative processes, which are predominantly under Nrf2 regulation, has been observed to be disturbed under many pathological circumstances.[2-6] Though considerable literature exists highlighting the mechanistic aspects of Nrf2 activation in response to oxidative stress, any explanation regarding Nrf2 insufficiencies observed during certain physiological and pathological conditions is still lacking. Here, we have shown that oxidative stress, which initially activates pro-survival antioxidant defenses, may be aggravated owing to signaling cues that suppress Nrf2-mediated transcriptional induction. Hence, the ultimate result following an oxidative insult, that is, survival or cell death, may depend upon the prevailing stress levels and the
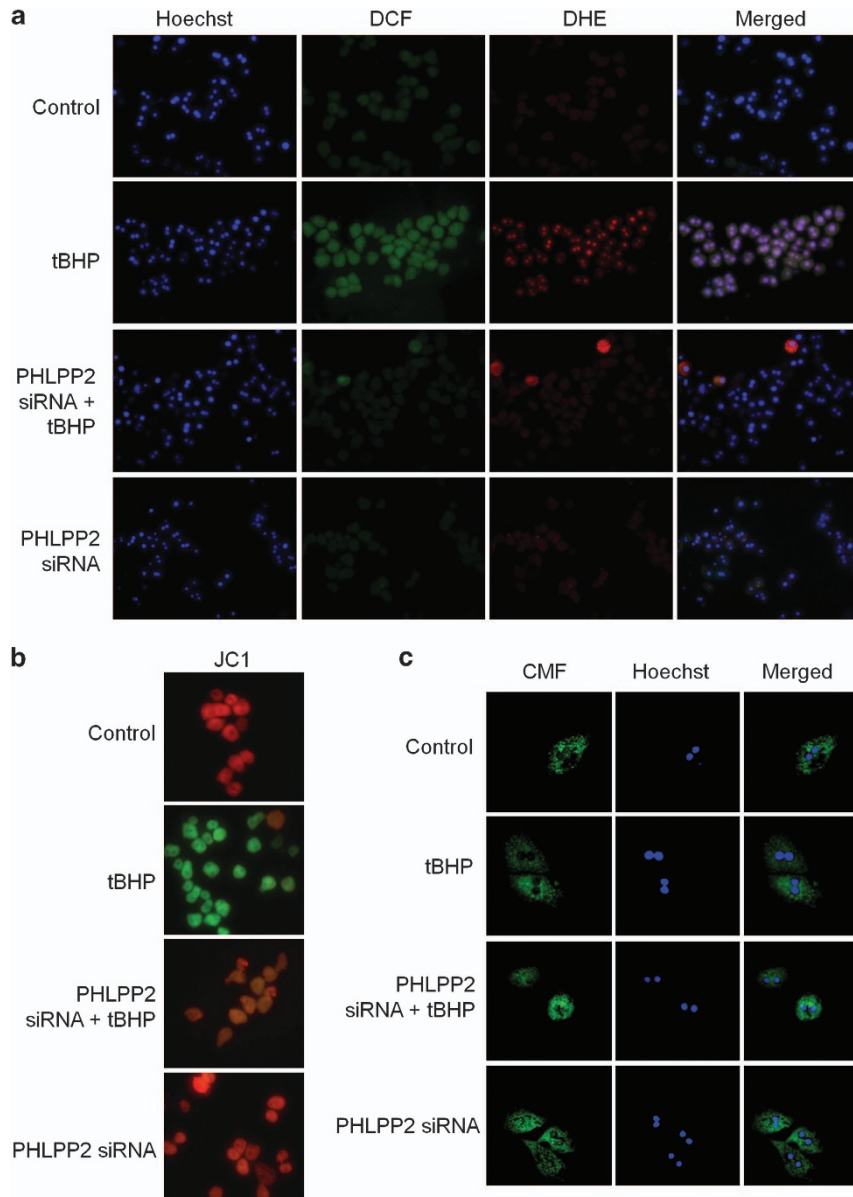
Figure 6 PHLPP2-silencing prevents tBHP-induced oxidative stress. Normal and PHLPP2-silenced hepatocytes were challenged with 250 μM tBHP for 90 min. (**a**) Fluorescent micrographs depicting tBHP-induced ROS generation as indicated by DCF staining (green) and DHE staining (red); (magnification $\times$ 10) (**b**) Alteration of mitochondrial membrane potential assessed by JC-1 staining of normal and PHLPP2-silenced hepatocytes (magnification $\times$ 20). The micrographs represent the images obtained after merging of red and green fluorescence channels. (**c**) Sub-cellular GSH levels assessed using fluorescence microscopy of CMFDA-stained normal and PHLPP2-silenced hepatocytes; (magnification $\times$ 63). The data are representative of at least three independent experiments

subsequent signaling arrays activated by them. Our data indicate that: (a) pathway regulating Nrf2 stability involves Akt–Fyn kinase crosstalk; (b) hepatocellular toxicity arising from tBHP-mediated oxidative stress is associated with Nrf2-suppression due to increased Fyn kinase activation; (c) tBHP-induced oxidative stress selectively downmodulates Akt activation at Ser473 residue; and (d) the selective site-specific deactivation of Akt, and hence Nrf2 stability, is controlled by PHLPP2. Thus, the study highlights a novel aspect in Nrf2 regulation with respect to PHLPP2 induction.

The PI3K/Akt pathway is implicated in a number of biological responses, such as apoptosis, cell growth, differentiation, calcium signaling and insulin signaling. Our study using LY294002, a PI3K inhibitor, reveals that the role of Akt in cell survival is linked to the promotion of Nrf2/ARE transcriptional regulation, which is brought to effect by checking GSK3β-mediated Fyn kinase activation. Fyn kinase, which is said to have key role in promoting Nrf2 degradation,[13,15,23] has been implicated in oxidative stress-associated functional insufficiencies[24,25] as well as apoptosis.[26–28] We observed that Fyn kinase inhibition using PP1 suppressed the endogenous ROS levels along with enhancement in stability and functional capacity of Nrf2. Not only this but also the dysregulation in Nrf2 signaling observed during hepatocytes death following tBHP treatment was seen as a consequence of Fyn kinase activation resulting from modulation of Akt pathway.
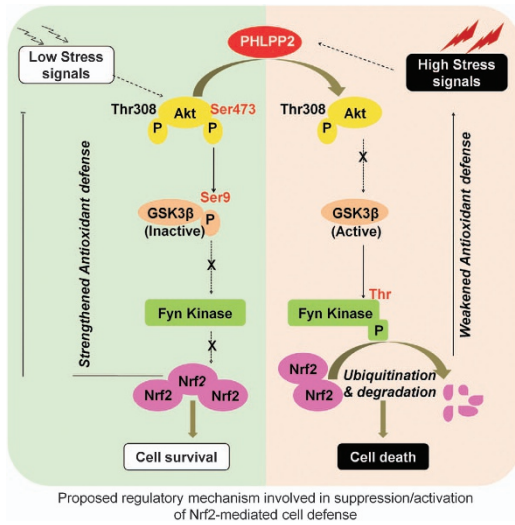
Figure 7 Cells' defense mechanism is triggered in response to rising levels of oxidative stress (due to endogenous/exogenous factors) in which Nrf2 regulation has a key role. A probable mechanism is that enhanced ROS generation disturbs redox balance and sends signals to the Nrf2 co-ordinated defense system via pathways involving Akt (left panel). Activated Akt inactivates GSK3β by phosphorylating its Ser9 residue. This results in inactivation of Fyn kinase which relieves ubiquitination-mediated Nrf2 suppression and thereby reinforces cell defense mechanism. PHLPP2 is a phosphatase that exclusively dephosphorylates Akt at its Ser473 residue. An event of toxic/oxidative insult may trigger signaling pathways leading to PHLPP2 induction that selectively downmodulates Akt Ser473 phosphorylation (right panel). This lifts the repression imposed by Akt on GSK3β activity, which phosphorylates and hence activates Fyn kinase resulting in Nrf2 degradation. Weakened cellular defense response further aggravates the stress levels that may lead to bio-molecular degeneration and ultimately cell death

An important finding of this study is the selective down-modulation of Akt phosphorylation at Ser473 residue during tBHP-induced hepatocyte death. Contrary to the common belief, necessitating phosphorylation of both Thr308 and Ser473 for Akt activation, increasing numbers of studies now indicate that selective phosphorylation of Akt, at either of its two residues, may activate Akt and generate distinct physiological response depending upon the specificity for its downstream substrates.[29] There are instances where distinct stimulants have been found to result in site-specific modulation of Akt phosphorylation.[30,31] Studies show that alteration in Akt activity through downmodulation of phosphorylation at Ser473 (independent of phosphorylation at Thr308 residue) renders the cell vulnerable to apoptosis.[32,33] Study by Kuo et al.[34] has demonstrated that selective dephosphorylation of Thr308, without an alteration in Akt(Ser473) phosphorylation, inhibited cell proliferation, but the effect was attributed to growth arrest and not increased cell death. Hence, while selective activation of Thr308 is observed to have a key role in promoting cell survival or proliferation,[29,34,35] a reduction in Ser473 phosphorylation seems to be necessary for establishment of apoptotic response. Moreover, GSK3β, a downstream target of Akt which is said to be involved in oxidative stress-mediated cell death,[36] is observed to be uniquely dependent on Akt phosphorylation at Ser473.[33,37] Although we detected a decline in phospho-Akt(Ser473) levels, no change was observed with respect to phosphorylation at Thr308 during tBHP stress (Figure 4a). As in the experiment

using Fyn kinase inhibitor PP1, we particularly observed decrease in phosphorylation at Thr308 of Akt (Figure 3a), we may assume that the enhanced Fyn kinase levels during oxidant attack may exert some feedback regulation at Thr308 site of Akt. Interestingly, a previous report illustrates that phosphorylation of Akt at Ser473 somehow inhibits that at Thr308 residue.[38]

The pattern of site-specific Akt activation conjures the involvement of upstream regulators of this kinase. PHLPP is a relatively recent addition to the milieu of signaling moieties regulating cellular homeostasis. Akt is one of the prime substrate of PHLPP, which, being a phosphatase, selectively dephosphorylates Akt at its Ser473 residue.[17] Owing to its decreased expression in several types of tumors, PHLPP has been defined as a tumor-suppressor gene. In a study by Liu et al.,[39] PHLPP2 was not only observed to be significantly lost in colorectal cancer patient specimens but an overexpression of the same also inhibited tumorigenic potential of colon cancer cells in nude mice. Incidence of polymorphism in the phosphatase domain of PHLPP2 in breast cancer cells[40] and repressed expression of PHLPP2 in Bcr-Abl expressing CML cells (chronic myelogenous leukemia cells)[41] as well as in non-small cell lung cancers[42] further highlight the relevance of PHLPP2 in modulating proliferative potential of the cell. In other words, the index of PHLPP2 expression within the cell may also be a measure of its susceptibility to death. A recent study reported that Atorvastatin (a drug used for the treatment of cardiovascular diseases) induced apoptosis in cardiac myxomas, a common primary tumor of the heart, by specifically enhancing phosphatase activity of PHLPP2 isoform of PHLPP.[19] Similar other reports[20,43] point toward pro-apoptotic functions of PHLPP2.

The role of PHLPP in regulating Akt activity in the liver is not much explored. PHLPP2 is not observed to be sufficiently expressed in un-induced rat liver;[44] however, DNA microarray analysis of liver tissue from lanthanum nitrate-treated rats revealed 23.4-fold induction of PHLPP2 transcript.[45] This is indicative of a possible role played by PHLPP2 in the progression of compromised liver pathophysiology. We are well aware that oxidative stress is a cardinal player in the inception, exacerbation or progression of many diseases, may they be of proliferative or of degenerative nature. Liver is a major site of xenobiotic transformation and, as a consequence, is often challenged with hepatocellular injury arising due to oxidant/antioxidant imbalances. We have already discussed the crucial role of PI3K/Akt pathway in containing ROS-mediated cellular damage via promotion of Nrf2-driven antioxidant defense. Given the ability of PHLPP2 to suppress Akt signaling, with this study we have also unveiled a previously unexamined role of PHLPP2 in regulating Nrf2 responses in the liver cells. We observed pronounced increase in both total and nuclear PHLPP2 levels together with significant decline in Akt(Ser473) phosphorylation upon tBHP exposure. PHLPP2-silencing not only restored normal Nrf2 signaling but also prevented mitochondrial depolarization and ROS generation in tBHP-exposed hepatocytes. Thus, our data confer convincing evidence that PHLPP2 may have a key role in determining the fate of cell by favoring apoptosis through selective suppression of Nrf2-regulated cellular defenses.

Increased basal activation of Nrf2, as has been observed in genetic analyses of many human tumors, allows the cancer cell to avoid the adverse effects of high levels of ROS and hence evade apoptosis.[46] In view of the loss of PHLPP2 function in many cancers, our investigation, which unveils a salient aspect of survival network describing PHLPP2–Akt–GSK3β–Fyn kinase–Nrf2 signaling axis, may explain the reason behind unhindered Nrf2 activation during cancer progression.

In summary, we show that Nrf2 insufficiency arising during oxidant attack may, at least in part, be a result of perturbed upstream signaling pathway regulating Nrf2 stability. We propose a mechanism by which PHLPP2 specifically dephosphorylates Akt at Ser473 residue, thereby lifting the regulation imposed by it on GSK3β, which in turn activates Fyn kinase that promotes Nrf2 degradation (Figure 7). The study identifies for the first time the critical function of PHLPP2 in regulating redox-sensitive Nrf2 signaling pathway, which could serve as a new target for developing strategies to manage pathological conditions exacerbated owing to oxidative stress. However, further insight into the multitude of signaling avenues in terms of mechanistic regulation imposed by PHLPP2 on cell survival need to be explored in order to minimize off-target effects.

Materials and Methods

Materials and reagents. All reagents are listed in Supplementary Materials and Methods.

Primary rat hepatocytes isolation, culture and treatment. Hepatocytes were isolated from 6- to 8-week-old Wistar rat through portal vein collagenase perfusion of liver as per the method of Seglen.[47] For attachment to collagen-coated surface, cells were cultured for 4 h in William's medium E supplemented with 50 nmol/l dexamethasone and 5% fetal bovine serum (FBS) in addition to 2 mmol/l glutamine and $1 \times$ anti-mycotic and anti-biotic solution. Thereafter, the cells were cultured in the same medium but without dexamethasone and FBS. Hepatocytes were treated with LY294002 (10–50 μM concentration range) to inhibit PI3K and PP1(5–25 μM concentration range) to inhibit Fyn kinase activity, both for a period of 30 min. For inducing oxidative stress in hepatocytes, exposure to 250 μM of standard oxidant tBHP was given for time periods ranging from 15 min to 3 h.

ROS detection. To measure intracellular ROS, cells were stained with 10 μM DCFH-DA (2′,7′-dichlorofluorescein diacetate) for 30 min before treatment. Fluorescence-activated cell sorting (FACS) was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). For fluorescent microscopic detection of ROS, hepatocytes were stained with 10 μM DCFH-DA and 5 μM DHE. Hoechst 33258 was used to stain nuclei and observed under Leica DMLB Fluorescence Microscope (Wetzlar, Germany).

JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide) staining. In order to assess alterations in mitochondrial membrane potential, hepatocytes were incubated with JC-1 at a final concentration of 2 μM at 37 °C through the 30 min time period of the experiment involving Akt and Fyn kinase inhibition. Nuclei were counterstained with Hoechst 33258 and observed under Leica DMLB Fluorescence Microscope.

Intracellular GSH estimation and localization. For GSH estimation, CellTracker Green CMFDA dye (5-chloromethylfluorescein diacetate; Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) was used. Cells were incubated with 2 μM CMFDA at 37 °C for 30 min, following which fluorescent microscopic detection was performed under Leica DMLB Fluorescence Microscope. Nuclei were stained using Hoechst 33258.

Enzyme activities. For methods followed to measure enzyme activities, refer Supplementary Materials and Methods.

Sub-cellular fractionation. Nuclear and cytoplasmic fractions were obtained following the NE-PER nuclear and cytoplasmic extraction protocol (Pierce Biotechnology, Rockford, IL, USA). Concentration of protein was determined using the bicinchoninic acid method.[48]

Immunoprecipitation and western blotting analysis. *In-vivo* ubiquitinated Nrf2 levels were estimated as described earlier.[49] To minimize non-specific precipitation, cell lysates were incubated with normal goat serum for 1 h before precipitation with Nrf2 antibody (developed in goat). For western blot transfer, BioTrace PVDF (polyvinylidene fluoride) membranes (Pall German Laboratory, MI, USA, USA) were used and visualized using the Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate Kit (Millipore Corporation, Billerica, MA, USA) on ImageQuant LAS 500 detection system (GE Healthcare, Upsala, Sweden). All western blotting images are representative of three independent experiments. The bands from western blotting were quantified by the ImageJ 1.47v software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence. Following treatment, cells were washed with cold 0.01 M PBS (pH 7.2) and fixed in 4% paraformaldehyde for 10 min. The cells were then washed with 0.05% glycine in PBS and then permeabilized with 1% Triton X-100 (v/v in PBS) for 15 min followed by overnight incubation with primary antibody at a dilution of 1:200 in PBS. The cells were rinsed thrice with PBS for 5 min each. This was followed by 1-h incubation in fluorescence-tagged secondary antibody at 1 : 500 dilution. Nuclei were counter-stained with Hoechst 33258 (5 μg/ml) for 5 min. Microscopic detection was performed under Leica DMLB Fluorescence Microscope.

siRNA transfection. Hepatocytes were cultured overnight in collagen pre-coated 24-well plate and transfected with 10 nM Silencer Cy3-labeled negative control siRNA using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Life Technologies Corp.) in agreement with the manufacturer's instruction to monitor transfection efficiency of isolated hepatocytes. Examination of hepatocytes 24 h after transfection with Cy3-labeled siRNAs revealed a near 70–80% transfection efficiency (Supplementary Figure S5a). Silencer Select predesigned siRNA against PHLPP2 was obtained from Ambion (Austin, TX, USA). Transfection was performed 24 h after plating using Lipofectamine RNAiMAX Transfection Reagent in agreement with the manufacturer's instruction in Opti-MEM Reduced Serum Medium (Invitrogen, Life Technologies Corp.). After 4-h incubation, the transfection medium was changed with serum supplemented William's medium E, and the hepatocytes were further cultured for an additional 48 h, following which stimulations by tBHP treatment (250 μM) were given for additional 90 min where required. Western blotting analysis was performed to confirm knockdown of PHLPP2 compared with negative control siRNA.

TransAM Nrf2-ARE binding assay. The Nrf2 DNA binding activity was measured using ELISA-based assay (TransAM kits, Active Motif, Carlsbad, CA, USA) following the manufacturer's instructions.

Statistical analysis. All computational calculations of quantitative data were performed using the Microsoft Excel program (Microsoft Office 2007, Microsoft Corporation (India) Pvt. Ltd., Gurgaon, India). Each experiment was repeated at least three times. The quantitative variables represented in histograms are expressed as mean ± S.E. Statistical comparisons between means of different groups were conducted by one-way analysis of variance followed by Tukey's *post hoc* test using the SPSS 14.0 statistical package (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when $P < 0.05$.

Conflict of Interest
The authors declare no conflict of interest.

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