Association of Gpx1 fluctuation in cell cycle progression

Mst. Khudishta Aktar[1,2], Md. Abdul Kafi[1] and Ravinder Dahiya[1*]

[1]BEST group, School of Engineering, University of Glasgow, United Kingdom

[2]Department of Life Science, Ewha Womans University, Seoul, Korea

[*]Corresponding author:

Ravinder Dahiya, PhD

Professor

School of Engineering

University of Glasgow

United Kingdom

Telephone: +44 (0)141 330 5653

Email: Ravinder.Dahiya@glasgow.ac.uk

Abstract

This research demonstrates fluctuation of glutathione peroxidase1 (Gpx1) throughout cell cycle progression with significant decreased expression at mitosis of HeLa cell. This was achieved with western blot (WB) analysis of target proteins from each phase of synchronized cells. The synchronizations were performed with double thymidine (T/T) for G1/S arrest and thymidine followed by nocodazol (T/N) for G2/M arrest. The G1/S arrested cells were released in fresh medium for 3, 6, 9, 10 and 15h to obtain cell at each phase such as Gap1 (G1), synthesis (S), gap2 (G2), mitosis (M) and gap1 (G1) phase, respectively for investigating Gpx1 expression throughout a complete cycle. The synchronizations were confirmed using fluorescence activated cell sorting (FACS) and WB analysis of phase specific markers. The fluctuations of Gpx1 expression were verified with universal protein actin and peroxiredoxin 1 (Prx1) which are stable throughout the cell cycle. Intriguingly, immunoblots showed the level of Gpx1 decreases at mitosis phase and increased during mitotic exit to G1 phase in HeLa cells, while Prx1 protein level remained constant. The fractionation experiments reveal that only the cytosolic Gpx1 was decreased while their levels at mitochondria remain constant. The heighest levels of mitochondrial ROS were measured in mitosis phase with FACS analysis using Mito sox indicating that antioxidant activity of Gpx1 for detoxifying excessive induced endogenous reactive oxygen species (ROS) in the mitosis phase could be the reason for such decreasing level. For unfolding the molecular mechanism of such decreased expression, the Gpx1 was investigated at transcriptional, translational and proteosomal level. The results revealed that translational mechanism is involve in the decreased expression rather than transcriptional or proteosomal degradation at mitosis phase. This finding supports that Gpx1 involved in the cell cycle progression through regulation of endogenous ROS. Based on this observation further research could uncover their possible association with the infinitive division of a cancer cell.

***Key words*:** Glutathione peroxidase, Cell division, Mitosis, Reactive Oxygen species, Antioxidant protein.

Introduction

Many bioengineering approaches have been focused on engineering of artificial platform to explore *in vitro* cell adhesion, proliferation and growth of tissues with functions equivalent to natural (Kafi et al. 2011, 2012). However, that has not been happened to date as the generation of excessive ROS remains as a challenge and the issue remains unattended. Manipulation of ROS scavenging mechanism could be appropriate step for resolving this issue. For this, the detail knowledge of natural antioxidant proteins involving the ROS scavenging mechanism is required. Therefore, this study focusses on the role of major ROS scavenger glutathione peroxidase (Gpx) for uncovering their association in cell cycle progression. A numbers of antioxidant proteins, such as Gpx, peroxiredoxin (Prx), superoxide dismutase (SOD) etc. are involved in maintaining intracellular homeostasis for optimum cell growth and function (Li et al. 2013; You et al. 2015). This homeostasis is maintained by their up- or down-regulation and by their depletion due to the intra/extra cellular ROS (Ray et al. 2012; Idelchik et al. 2017). The research dealing with uncovering roles of antioxidant proteins during cell cycle progression attracted much attention.

Gpx protein, known as selenoprotein, consists selenium in it's catalytic site (Lu et al. 2009). Several isoform of Gpx proteins have been reported until to date (Herbette et al. 2007). Among these, Gpx1 is abundant isoform, which involves in scavenging endogenous ROS using electron provided by reduced glutathione and ubiquitously expressed in the cytoplasm and mitochondria of all cell types (Espinosa-Diez et al. 2015). The association of Gpx1 over expression with buffering oxidative stress has been proven in both *in vitro* cell culture and *in vivo* genetic mouse models (Borchert et al. 2006, Powers et al. 2008).. The up regulation of nuclear and cytosolic Gpx in differentiating cells than proliferative cell during enterocytes differentiations has been reported (Speckmann et al. 2011). Most of these previous studies apply exogenous stimulation for the over expression of Gpx to explore their specific role, which is insufficient for describing about status of endogenously induced Gpx expression. Therefore, monitoring of endogenous expressed Gpx at various phases of cell division cycle requires for exploring the possibility of their association in cancer cell proliferation.

The mammalian cell follows a cascades of physiological events such as G0,, G1, S, G2 and Mitosis (M) for their proliferation and growth (Schorl et al. 2007). These cascades of events are influenced with the levels of endogenous ROS (Boonstra et al. 2004). In stressed condition, the excessive endogenous ROS causes disruption of several cascades of event in cell cycle progression (Barrera et al. 2012; Redza-Dutordoir et al. 2016). It has been reported that moderate level of ROS is required for G1/S transition during cell cycle Havens et al. 2006). However, excess level ROS disrupt such transition through activating apoptotic signaling (Circu et al. 2010).

Thus, regulation of levels of endogenous ROS is critical event for the smooth transition of a cell cycle. The antioxidant proteins with ROS scavenging ability is critically requires for maintaining their optimum level for cell growth. There are few studies reported such scavenging mechanism of Gpx that plays critical role for the maintenance of cellular homeostasis for the natural cell function (Wang et al. 2013). The removal of endogenous ROS by the over expression of Gpx has been reported to induce G0/G1 arrest (Onumah et al. 2009) by decreasing cyclin D1 and increasing in CDK inhibitory protein $p27^{kIPI}$. However, the expression pattern of Gpx and their ROS depletion mechanism has not been investigated all the other stages. The Gpx expression in mitotic stage is critical, because cell experiences stress at this phase resulting excess ROS generation (Wellen et al. 2010). Thus, investigation on Gpx expression and their role in regulation of ROS during the mitosis phase of mammalian cell division is critically required.

In this study, expression level of antioxidant protein throughout the cell cycle progression was monitored for establishing their involvement in cell growth and proliferation. We have reported detail expression patterns of Gpx1 protein in HeLa cell at their various stages of division cycle. For this, HeLa cell line was synchronized at synthesis (G1/S) and mitosis (G2/M) stages using double Thymidine (T/T) and Thymidine/Nocodazole (T/N) block, respectively. The synchronization was verified using FACS and western blotting (WB) analysis of phase specific proteins such as CyclinA1,Cyclin B, Cyclin D1, Cyclin E1, P27, Phosphohistone 3 (p-HH3). The levels of Gpx1 protein expression were investigated using (WB) where the fluctuations are measured with respect to the universal protein actin. The G2/M arrested cells were released in fresh medium for monitoring the levels of Gpx1 with respect to their release period. The G1/S arrested cells were released in fresh medium for 3, 6, 9, 10, and 15h for obtaining stage specific endogenously expressed Gpx1. A fractionation experiment of synchronized cells was performed for observing the local fluctuation of Gpx1 for further confirmation of their association with stage specific expression. This research demonstrates the levels of Gpx1 fluctuate throughout the cell division cycle with a lowest intensity at mitosis phase. The research also monitored the ROS levels at the corresponding synthesis, mitosis and G0 arrest for establishing the relations with Gpx1 expressions. The lowest level of Gpx1 and the highest levels of ROS at mitosis could be due to antioxidant activity of Gpx1 for scavenging excessive ROS, which is required for smooth progression cell division cycle.

Materials and Methods

Chemicals and Reagent Primary antibodies used in this study are: Rabbit anti-Gpx1 (AbFrontier; Seoul Korea); Rabbit anti-cyclinB1 (Santa Cruz Nanotechnology); Rabbit anti-phospho-Histone

H3(Millipore); Mouse anti-Actin; rabbit anti-peroxiredoxin (abfrontier), Cyclin A anti-rabbit Cycloheximide (Sigma), MG132 (Sigma).

Cell Culture Human cervice cancer (HeLa) cells were maintained at 37°C in a 5% CO_2 incubator and cultured in Dulbecco's modified Eagle's Medium (DMEM) which contain 4500 mg/L D-glucose and L-glutamine with 10% Fetal bovine serum 1% penicillin and streptomycin. Cells were feeded twice in a week and sub-cultured from 90% confluent plate. Cells from third passage were used for experiment throughout the research.

Cell Synchronization HeLa cells were cultured at 37°C in a 95% air, 5% CO_2 and 70% humid condition in DMEM supplemented with 10% heat inactivated fetal bovine serum, 1% penicillin, and 100 mg/ml streptomycin. Synchronization of HeLa cells at G1/S phase were carried out using double thymidine block (Fang et al. 1998). Briefly, cells were incubated with 2 mM thymidine for 18h. After washing with PBS, cells were incubated in fresh culture medium for 8h. Thymidine was added into the culture medium (to a final concentration 2 mM) and incubates for another 18h. Cells from G1/S phase were released by replacing thymidine treated culture medium with freshly prepared medium. The G2/M arrested cells were achieved by incubation in thymidine treated medium for 18h. Then, cells were washed with PBS thrice prior to the nocodazole (100ng/ml) for 10h.

Sub Fractionation of Mitochondria Cytoplasm and mitochondria were isolated by fractionation methods using different spinning speed. Synchronized (G1/S and Mitosis arrested) Hela cells were homogenized in ice-cold buffer (70mM Sucrose, 1mM EDTA, 2mM Hepes, pH 7.4 220mM Manitol). The homogenates were centrifuged at 1000g for 10min at 4^0C. The resulting supernatant was then centrifuged at 12000g for 15 min at 4^0C to obtain a crude heavy mitochondrial pellet. The supernatant was collected as cytoplasmic fraction. The crude heavy mitochondrial pellet was washed once for further purification with same buffer. The pellet and supernatant were subjected to Western blotting analysis.

Fluorescence Activated Cell Sorting For the analysis of cell cycle, 5×10^5cell/ml were washed twice with ice cool PBS and place overnight at 4^0C in 70% ethanol for fixing. The fixed cell were stained with 1ml sol'n containing RNase (sigma) of 50µg/ml and propidium iodide (sigma) of 50µg/ml. Cells were incubated at least 30min at 37^0C and analyzed using the FACS caliber flow cytometer (BD science). To check the levels of super oxides the cell expressing Mito sox was analyzed at the excitation of 488nm.

Western Blot Analysis Cell lysates were prepared in lysis buffer 50mM Tris (pH 7.7)150mM NaCl .5% NP-40 10% glycerol1mM DTT (just add before use) Protease inhibitor (just add before

use) DDW. Supernatant after centrifugation was recovered and protein content were quantified by the Bradford assay (Bio red Laboratories). Total proteins were separated by electrophoresis on 12 and 14% SDS-polyacrylamide gels depending on size of target protein being investigated. The proteins were electro-blotted onto nitrocellulose membranes, probe with Primary antibody overnight, and re-stripped in secondary antibody after washing with PBS.

Quantitative Real-Time PCR Total RNAs were isolated with Trizole Reagent and reverse transcribed with a reverse transcription (RT-PCR) kit (Applied Biosystems) according to manufactures instructions. Power SYBER Green PCR master mix (Applied Biosystems) was used to quantify the mRNA expressions. The primers used for quantitative RT-PCR were as follows: Gpx1 sense 3' CAA CCA GTT TGG GCATCA G 5' antisense 3' GTTCACCTCGCACTTCTCG 5'.

Statistical Analysis All experiments were repeated at least three independent experiments and quantitative data were presented as mean S.E.M of triplicate deternminations from representative experiments. Data were analyzed using Student st-test on sigma plot 10 software and deriving the p-value to access the statistical significance. All western blots were done at least three to show reproducibility.

Results

Synchronization of HeLa cell for Monitoring ROS Scavenger Proteins For establishing the role of Gpx1 at mitotic phase initially two important ROS scavenger Gpx1 and Prx1 were critically monitored in the various phases of the cell cycle. For monitoring Gpx1 and Prx1 expression, HeLa cells were synchronized at synthesis (G1/S), mitosis (G2/M) and resting phase (G0), whereas unsynchronized cells were maintained in parallel as control. The synchronizations were confirmed with florescent activated cell sorting (FACS) (Fig. 1a) and immunoblot analysis of phase specific proteins, such as cyclin B and p-HH3 as mitotic marker,, CyclinE1 as G1/S marker and P27 as G0 marker (Fig.1b). Western blot analysis from G1/S, G2/M, G0 and unsynchronized Hela cells reveals that Gpx1 protein level fluctuates during cell cycle progression, whereas Prx1 level was constant (Fig. 1b). The highest Gpx1 expression was observed at G1/S and decreases at G2/M and G0 phase. Being seleno protein, Gpx1 expression is known be depended on selenium supplementation (Goldson et al. 2011). The decreased expression of Gpx1 at serum starved G0 phase is obvious since serum is the only source of selenium in the in vitro system (Mehdi et al. 2016). However, the decrease level of Gpx1 at G2/M was independed to selenium concentration since at this stage selenium level similar to G1/S. So, other hidden cause might be involved with the decreasing Gpx1 at G2/M phase. To uncover such hidden fact the

detail investigation of Gpx1 expression at various periods of release from G2/M arrest was performed later in this work.

Gpx1 in Cells Released from Mitosis phase The gradual Gpx1 upregulation in cell released from mitotic phase was investigated for unfolding the hidden fact behind the mitotic decrease. This was performed with monitoring Gpx1 protein in cell release from G2/M phase in a time dependent manner. For this, Hela cells were synchronized at G1/S phase by T/T treatment, G2/M phase by T/N treatment followed by the release in fresh media for 2, 5 and 10h. The synchronization was confirmed by FACS analysis (2a) and by detecting phase specific marker protein such as cyclin B and p-HH3 for G2/M, Cyclin E1 for G1/S, Cyclin D1 for G1 and Cyclin A1 for S phase marker (2b). Cyclin B, a well-known mitotic marker highly expressed in T/N blocked cells and protein levels were decreased gradually with release period from T/N block (Fig. 2b) (Gavet et al. 2010). p-HH3 another important mitotic marker protein was only expressed in T/N arrested cells (Nielsen et al. 2013). Whereas, the lowest expression of Gpx1 measured from the G2/M phase and followed by gradual increased expression were noticed from cells released from G2/M phases (Fig. 2b). Densitometric analysis revealed that expression of Gpx1 protein levels were 1.5 fold higher in G1/S (T/T) arrested cells compare to mitotic cells and the levels were continuously increased in cell released from mitosis phase. Whereas, a stable expression of Prx1 were observed all phases throughout the cell cycle (Fig. 2c). This time dependent gradual increase of Gpx1 expression cells release from mitosis phase indicated their lowest expression in the G2/M phase. However, the antioxidant activity of the Gpx1 protein in the detoxification of endogenous ROS for the smooth transition of cell cycle could be the prime cause of such decrease level of Gpx1 at G2/M phase. For establishing this, ROS scavenging activity correlation between the levels of Gpx1 and endogenous ROS needs to be investigated form the synchronized phases of the cell cycle.

ROS Level Throughout Cell Cycle To prove the ROS scavenging activity of Gpx1, ROS levels was measured in synchronized HeLa cells with Mito sox using flow cytometry analyzer (Li et al. 2011). For this, cells were released from mitotic arrest for 2 and 5h for achieving G1 and G/S arrest and confirm with phase specific protein cyclin B, Cyclin D1 and Cyclin E1 as presented in figure 3c. ROS levels measured in synchronized HeLa cells using FACS are presented in Fig 3a. The levels of ROS levels in mitotic cells were higher than that of G1/S cells and decreases after exit from mitosis phase to G1/S phase (Fig. 3a,b). This result suggested that ROS levels fluctuate throughout cell cycle progression reversely as does for Gpx1. In fact, their highest level at mitosis phase is obvious because cell experiences severe oxidative stress at this stage (Wellen et al. 2010). Gpx1 could be utilized in the ROS depletion mechanisms and results their decreased levels at

mitosis phase. However, this mitotic decrease of Gpx1 was further critically investigated later in this work.

Gpx1 at All Phases of Cell Cycle The mitotic decrease of Gpx1 protein was further confirmed with monitoring their expression at all phases of a cell cycle. For this, HeLa cells were release from G1/S arrest (T/T block) for 3, 6, 9, 10 and 15h for obtaining G1/S, S, G2, M and G1, respectively and Gpx1 expressions were monitored at each of those phases for revealing their fluctuations throughout the cell cycle. Western blot analysis in Figure 4b showed Gpx1 protein expression were decreased only at mitosis phase (10h release from T/T block) and approximately protein levels were 4 fold increased at G1/S arrested cells compare to G2/M arrested cells. No significance difference was found among other phases of the cell cycle. Synchronizations were confirmed by FACS (Fig. 4a) and western blot analysis of specific marker protein p-HH3 and cyclin B, Cyclin D1, Cyclin E1, Cyclin A1 as stated before (Fig. 4b) (Gavet et al. 2010; Nielsen et al. 2013). Relative band intensity of Gpx1 signified the difference of protein level between mitosis and other phases of cell cycle (Fig. 4c) suggesting that Gpx1 protein levels decreases only at mitosis phase.

Local Fluctuations of Gpx1 It is known that Gpx1 localizes both in the cytoplasm and mitochondria of a living cell (Kryukov et al. 2003; Diamond et al. 2015). Hence, investigation is still required to figure out whether the cytoplasmic or mitochondrial Gpx1 are involves in the ROS depletion mechanism and eventually responsible for the decreased level at mitosis. We determine localizations of Gpx1 by separations of total lysates, cytoplasm and mitochondria followed by immunoblot analysis. Synchronizations were confirmed by the detection of p-HH3 and Cyclin E1 using total lysates (Fig. 5a). Cytoplasm, mitochondria and total lysates were isolated from G1/S and G2/M arrested cells by fractionation method and confirm by corresponding protein detection such as cytoplasmic protein Prx2 and mitochondrial protein Prx3. A constant level Gpx1 were observed in whole cell lysates, cytoplasmic and mitochondria (Fig. 5a) at G1/S in G1/S phase (Fig. 5a). A low level Gpx1 from total lysate and cytoplasm were measured from the Mitotic arrest whereas its level at mitochondria was similar to G1/S (Fig. 5a). This difference in Gpx1 expression was significant in cytoplasm and total lysate, whereas non-significant in mitochondria as observed from densitometric analysis (Figure 5b). This observation reveals that only the cytoplasmic Gpx1 proteins are decreased at mitosis phase. The association of cytoplasmic Gpx1 in ROS scavenging mechanism could be the reason which could be figure out in future.

Molecular mechanisms of Gpx1 fluctuations during cell cycle progression Quantitative (Q)-RT-PCR was performed to investigate the level of Gpx1 mRNA during cell cycle progression.

RNAs were isolated from G1/S (T/T and T/N 10h release) and mitosis (T/N) phase arrested HeLa cells for the Q-RT-PCR analysis. Q-RT-PCR analysis revealed that Gpx1 mRNA is abundant in G1/S and mitotic phase (Figure 6a). Although, Gpx1 mRNA level is abundant in both phases, but their protein level decreased at mitosis phase. This indicates Gpx1 protein level might be regulated by post transcriptional mechanism. To prove this, we investigated proteosomal degradaion pathway of Gpx1 protein at mitosis phase using a well-known protease inhibitor MG132. As shown in figure 6b, cyclinA degradation was inhibited by MG132 at mitosis phase however such inhibition did not occur in case of Gpx1. The relative band intensity of Gpx1 shows that Gpx1 does not decrease at mitosis phase by proteosomal degradation pathway (Fig.6c). We also investigated the translational mechanism during mitotic exit for unfolding the mechanism involves behind the reduction of Gpx1 in the mitosis. For this, cyclohexamide (CHX) was used to inhibit translation of Gpx1 in cells released from T/N block. Gpx1 expression was inhibited in CHX treated cells whereas such inhibition did not occur in non-treated released cell (Fig.6d,e).. The results revealed that CHX successfully inhibit Gpx1 induction in T/N released cell which indicates that mitotic decreased Gpx1 was gradually increased in T/N released cell due to translational mechanism.

We also investigated the mechanistic phenomenon behind differences in expression patterns of Gpx1 and Prx1 proteins regardless of their antioxidant activity. For this, we investigated the turn-over rate of both the antioxidant protein Gpx1 and Prx1 in Hela cells using translation inhibitor CHX for various time period. As our expectation, result reveals that CHX was able to inhibit Gpx1expression within 6h where as Prx1 protein expression was stable (Fig.6f,g). This result suggested that turn-over rate of Gpx1 is shorter than Prx1, which is responsible for the variations in their expression pattern at mitosis phase.

Discussion

In this study, fluctuation of Gpx1 expression was observed throughout cell cycle progression with the highest level at synthesis (G1/S) which decreases as progressed towards mitosis phase (G2/M) and reach the lowest level at G0 phase. Initially, we assumed two reasons behind these variations

in Gpx1 expression; first reason is the role of selenium (Se) containing serum-supplemented medium in the G1/S phase and second is the antioxidant activity of Gpx1 for depletion of excessive ROS in the mitotic phase. According to first assumption, the highest Gpx1 expression at G1/S was obvious because of the presence of Se in serum-supplemented growth medium and eventually the lowest at G0 phase because of the absence of Se serum free starving medium (Neve 1995). However, this hypothesis is supporting the differences in the expression of Gpx1 between G1/S and G2/M phase since serum-supplemented medium were both the cases. According to the second assumption, the decreased Gpx1 is due to their antioxidant activity for scavenging excessive ROS generated in the mitotic phase (Lubos et al. 2011; Hugo et al. 2018). In supporting either of these possibilities, further experiments were designed to confirm gradual upregulation of Gpx1 in cells released from G2/M arrest in a time dependent manner. This research reveals that Gpx1 was increased with the released period indicating that selenium dependent stimulated expression effect was insignificant. Therefore, the gradual increasing trend of Gpx1 expression with the released period indicating that their antioxidant activity is the principal reason behind the decreased level of Gpx1 at the G2/M phase. The activity of Gpx1 in mitochondrial ROS scavenging is proven phenomena describe elsewhere (Lubos et al. 2011;Ray et al. 2012). The oxidative stress induced superoxide radicals (O2-) is neutralized to water through a two-step process involving SOD in a first step and Gpx1 in the second step (Raha et al. 2000; Li et al. 2013). Any impairment of this process results generation of hydroxyradicle ($^{-}$OH) and peroxynitrite ($ONOO^{-}$) which are also removed by Gpx1 by converting it to the lipid alcohol and nitrite (NO2), respectively (de-Haan et al. 2011). Thus, majority of expressed Gpx1 is utilized for the depletions of excessive ROS generated in the G2/M phase for the smooth progression of cell cycle.

The association of ROS in the mitotic decreased level of Gpx1 was further supported by revealing the highest level of ROS with corresponding lowest Gpx1 at mitosis phase of a cell cycle as measured in this work. The fluctuations of ROS levels all through the cell cycle are natural phenomenon in natural cell cycle because a minimum levels of ROS is requires for the transition of specific cell cycle phases (Havens et al. 2006). Ibañez and coworkers observed Gpx1 is critical for the cell cycle progression (Ibanez et al. 2011). They observed cells are arrested at G1/S when cell exposed to abundant antioxidant proteins proving that the antioxidant protein needs to be at minimum levels for smooth transition of G2/M phase. These observations suggested that Gpx1 fluctuation requires for cell cycle progression. Such fluctuation of Gpx1 happened through three different molecular mechanisms such as proteosomal degradation, transcription and translation (Lubos et al. 2011). An in vivo study reports that Gpx1 protein levels and activity decreased by translational inhibition although there was abundant transcript (Handy et al. 2009) suggesting that

translational rather than transcriptional mechanism regulates expression pattern of Gpx1 during cell cycle progression. Regardless of similar antioxidant activity, expression patterns of Gpx1 and Prx1 are dissimilar because of their different turn-over rates. We observed Gpx1 expression turn-over occured within 6h whereas Prx1 expression remains stable indicating the shorter turn-over rate of Gpx1. This short turn-over rate results the decreased level of Gpx1 expression at mitosis.

Fractionation experiment showed that this mitotic decrease was only due to the decrease of cytosolic Gpx1. The mitochondrial Gpx1 remains unchanged regardless the phases of a cell cycle. This observation supports that mitotic decrease is only due to their utilization in scavenging ROS mechanism (Ighodaro et al. 2017). The mitochondrial Gpx1 indicated there accumulation and active participation in the oxidative stress induced ROS depletion mechanism (Handy et al. 2009). Therefore, post-translational uses of the Gpx1 would be the only case for the mitotic decrease. Previous study reported that cell employs their natural homeostasis mechanism through endogenously expressed catalytic proteins for maintain the adequate ROS level (Ludke et al. 2017). It is already established that many antioxidant proteins involves with depletion endogenously induced ROS for the completion mitotic cell division (Waris et al. 2006; Han et al. 2018). The decreased level of Gpx1 expressions is a clear indication of their involvement in the catalysis of endogenous ROS for maintaining their adequate level for a smooth progression of a cell cycle. Thus, Gpx1 involves in the cell division mechanism through the regulation of ROS level, which is critical phenomenon for the onset of mitosis phase of a cell division cycle.

Conclusion

This research demonstrates the fluctuations of Gpx1 protein during cell division, which is critically required for the maintenance of intracellular homeostasis through ROS depletion for the cell cycle progression. Gpx1 was remarkably decreased at the G2/M phase compare to G1/S phase in synchronized HeLa cell. A time dependent induction of Gpx1 was observed in cells released from G2/M phase. Reduction of Gpx1 exclusively observed only at mitosis phase suggesting utilization of Gpx1 at mitosis phase for detoxifying excessive endogenous ROS. In addition, fractionation experiment was further confirmed the reduction of Gpx1 in cytosol of cells arrested at mitosis phase. The similar level of Gpx1 expression was observed at G1, S, G2, except M phase is proving their utilization with the ROS detoxifying mechanism. This observation was further supported with highest ROS levels as measured in this work. This research also demonstrates that the mitotic decrease of Gpx1 was only at cytosol, while their presence in mitochondria indicating their accumulation for antioxidant activity. Translational mechanism rather than transcriptional or proteosomal degradation is involve in this decreased expression of Gpx1 at mitosis phase. The mitosis phases experiences severe oxidative stress, which induced

excess endogenous ROS mitochondria in proliferative cells like cancer cell. Therefore, the level of Gpx1 is critical for the maintenance of cell cycle for the generation of new cell population through the regulation of ROS. Further research could be focused for unveil specific mechanisms of Gpx1 dependent ROS regulation for exploring the possibilities of cancer cell regulation.

Author Contributions

M.K.A. involved in designing and performing the research work; M.K.A, M.A.K, and R.D. involves writing and formatting the manuscript. All the authors reviewed the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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Figure legend

Figure 1. Expression pattern of antioxidant protein throughout cell cycle progression (a) FACS analysis of synchronized HeLa cells. (b) Western blot analysis of whole cell lysates of synchronized HeLa cells. G1/S arrested HeLa cells were collected by double thymidine treatment (T/T), mitosis by thymidine followed by nocodazole (T/N) treatment and G0 phase cells were collected by serum starvation for 24 h.

Figure 2. Glutathione peroxidase protein levels decreases at mitosis phase in Hela cells (a)FACS analysis of synchonized HeLa cells. (b) Western blot analysis of whole cell lysates of synchronized HeLa cells. Cells were synchronized at G1/S phase by double thymidine block (T/T), mitosis phase by thymidine followed by nocodazole block (T/N) and then cells were grown in fresh media for 2, 5,and 10 hour after nocodazole block (c) Densitometric analysis of Gpx1 protein expression.

Figure 3. Intracellular H_2O_2 increased at mitosis phase (a) HeLa cells expressing hyper-mito were arrested at mitosis by thymididne/nocodazole block and release to G1 and G1/S phase by shaking off with fresh media for indicated time. The levels of H_2O_2 were measured by flow cytometry. The H_2O_2 levels were measured by quantifications of relative DCF intensity. (b) Relative intensity was converted to graph. Bar indicated the standard error. (c) Western blotting analysis of synchronized HeLa cells.

Figure 4. Fluctuations of Glutathione peroxidase throughout the cell cycle progression (a) FACS analysis of synchronized HeLa cells (b) Immunoblot analysis of whole cell lysates of all G1/S, S, G2, M and G1 phase arrested HeLa cells by double thymidine block (T/T). Cells were grown in fresh media for 3, 6, 9, 10 and 12 hour after double thymidine treatment (c) Densitometric analysis of Gpx1 band intensity. **p ≤0.01% and * p≤≤ 0.05%. Data represents means +SD of three independent experiments.

Figure 5. Cytoplasmic Gpx1 decrease at mitosis phase. (a) Immunoblot analysis of total lysates, cytoplasmic and mitochondrial fractions of G1/S and mitosis arrested HeLa cells. Phospho histone and Cyclin E1 were detected using total lysates. (b) Densitometric analysis of Gpx1. Immunoblot analysis of total lysates, cytoplasmic and mitochondrial fractions collected from 3 days cultured HeLa cells in the presence of selenium. Densitometric analysis of Gpx1. * p≤ 0.05% ** p≤ 0.01%. Data represents means +SD of three independent experiments.

Figure 6. Molecular mechanisms of Gpx1 fluctuations during cell cycle progression. (a). mRNA level of Gpx1 was quantified by RT-PCR analysis. Total RNAs were extracted from

double thymidine (G1/S phase) and thymidine nocodazole arrested (mitosis phase) HeLa cells. Total RNAs were analyzed by Q-RT-PCR analysis with normalization against 36B4 (b) HeLa cells were synchronized at G1/S phase by double thymidine block, Mitosis by thymidine followed by nocodazole block with or without MG132. Protein levels were analyzed by western blot analysis. Densitometric analysis of Gpx1 protein band intensity (c). (d) HeLa cells were synchronized in G1/S phase by double thymidine (T/T) block and mitosis phase by thymidine nocodazole (T/N) block and after nocodazole treatment cells were grown in fresh media for 10 hr with or without cycloheximide and subjected to immunoblot analysis.(e) Densitometric analysis of Gpx1 protein band intensity. * $p \leq 0.05\%$. Data represents means +SD of three independent experiments. (f) HeLa cells were exposed to cycloheximide for 0, 2, 4, 6 hours. Western blot analysis of whole cell lysates of cycloheximide treated HeLa cells. (g) Cyclin A, Gpx1, and Prx1 intensity were quantified by densitometric analysis. * $p \leq 0.05\%$ level of significance showed between 0 and 6h CHX exposure . Data represents means +SD of three independent experiments.

Figure 1

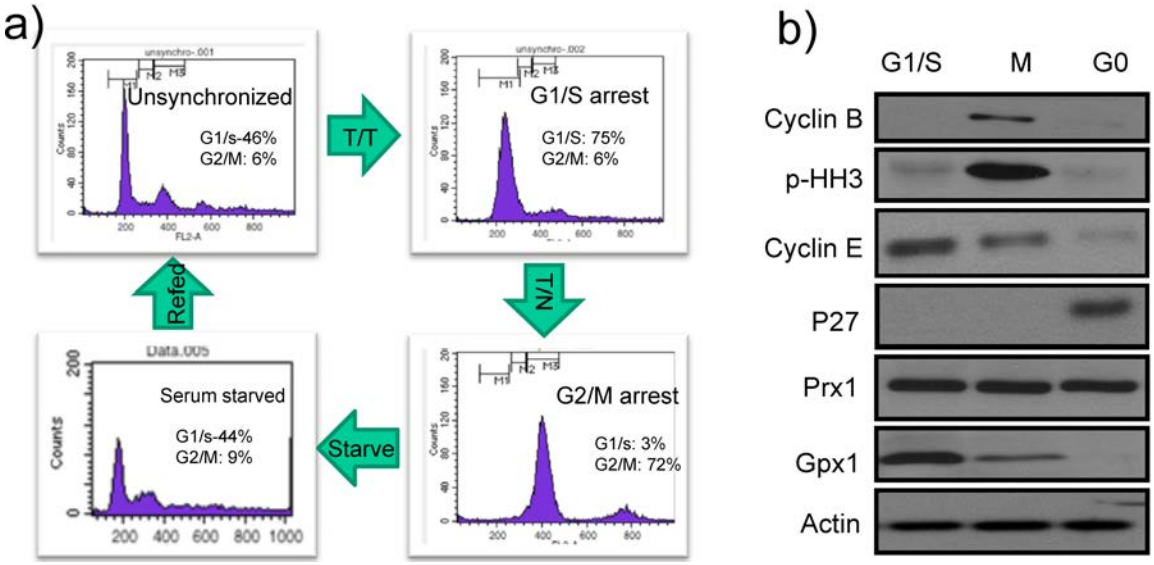

a)
Unsynchronized
G1/s-46%
G2/M: 6%
T/T
G1/S arrest
G1/S: 75%
G2/M: 6%
T/N
G2/M arrest
G1/s: 3%
G2/M: 72%
Starve
Serum starved
G1/s-44%
G2/M: 9%
Refed
b)
G1/S
M
G0
Cyclin B
p-HH3
Cyclin E
P27
Prx1
Gpx1
Actin

Figure 2

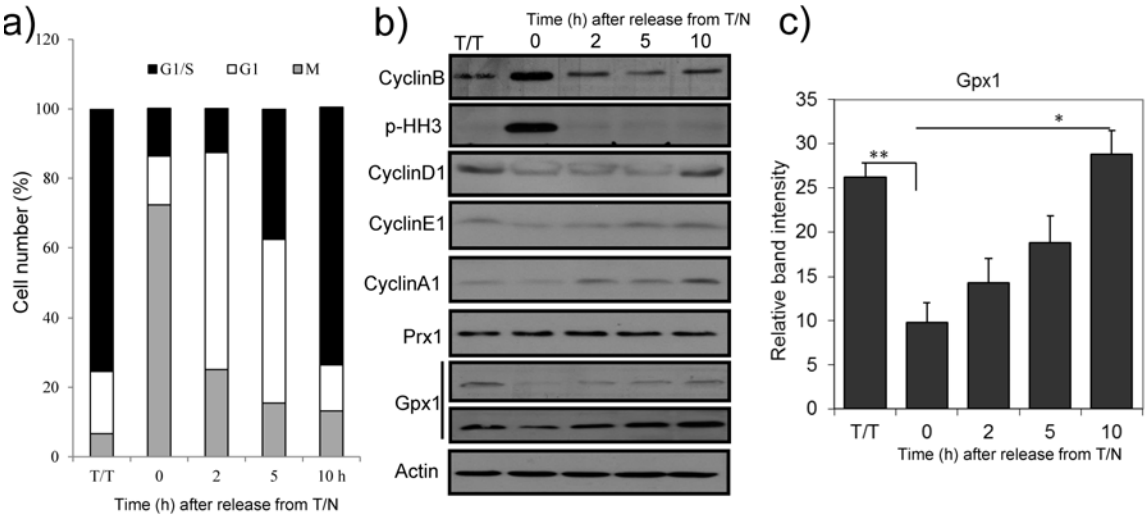

a)
120
100
80
60
40
20
0
Cell number (%)
G1/S
G1
M
T/T
0
2
5
10 h
Time (h) after release from T/N
b)
Time (h) after release from T/N
T/T
0
2
5
10
CyclinB
p-HH3
CyclinD1
CyclinE1
CyclinA1
Prx1
Gpx1
Actin
c)
Gpx1
35
30
25
20
15
10
5
0
Relative band intensity
**
*
T/T
0
2
5
10
Time (h) after release from T/N

Figure 3

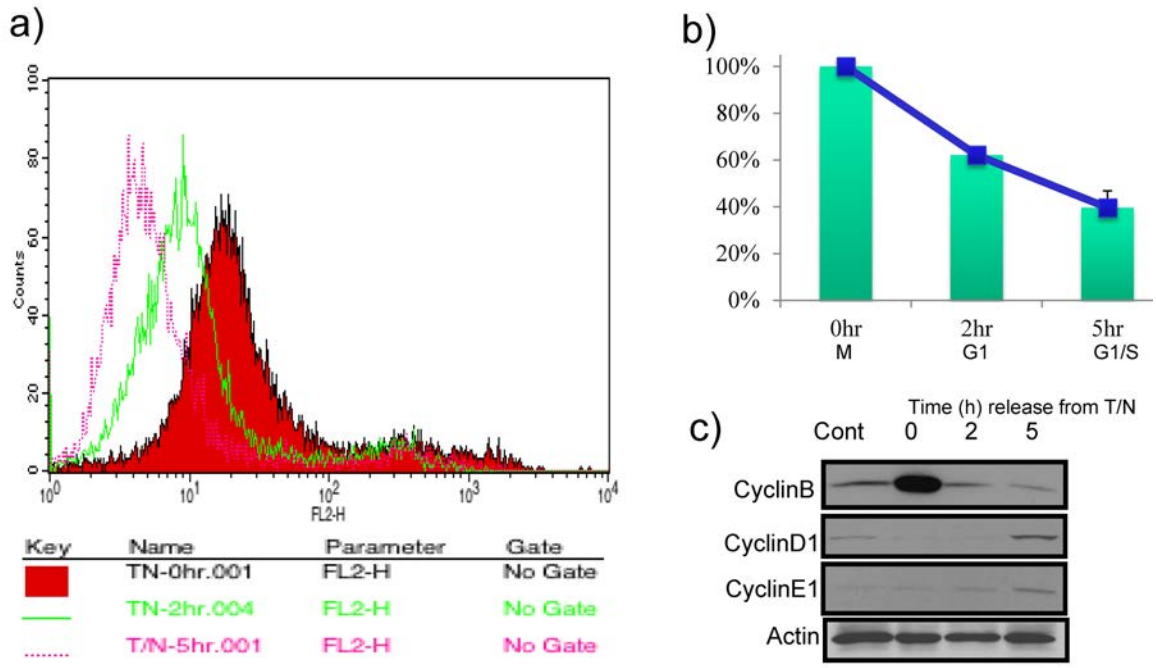

a)
Counts
FL2-H
Key
Name
Parameter
Gate
TN-0hr.001
FL2-H
No Gate
TN-2hr.004
FL2-H
No Gate
T/N-5hr.001
FL2-H
No Gate
b)
100%
80%
60%
40%
20%
0%
0hr
M
2hr
G1
5hr
G1/S
c)
Time (h) release from T/N
Cont
0
2
5
CyclinB
CyclinD1
CyclinE1
Actin

Figure 4

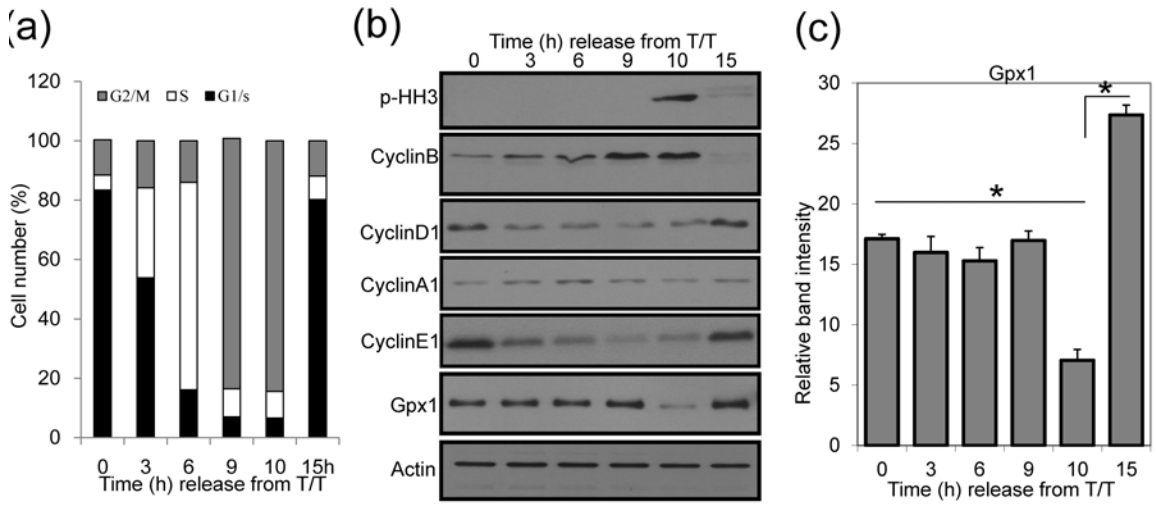

(a)
G2/M
S
G1/s
Cell number (%)
120
100
80
60
40
20
0
0 3 6 9 10 15h
Time (h) release from T/T
(b)
Time (h) release from T/T
0 3 6 9 10 15
p-HH3
CyclinB
CyclinD1
CyclinA1
CyclinE1
Gpx1
Actin
(c)
Gpx1
Relative band intensity
30
25
20
15
10
5
0
*
*
0 3 6 9 10 15
Time (h) release from T/T

Figure 5

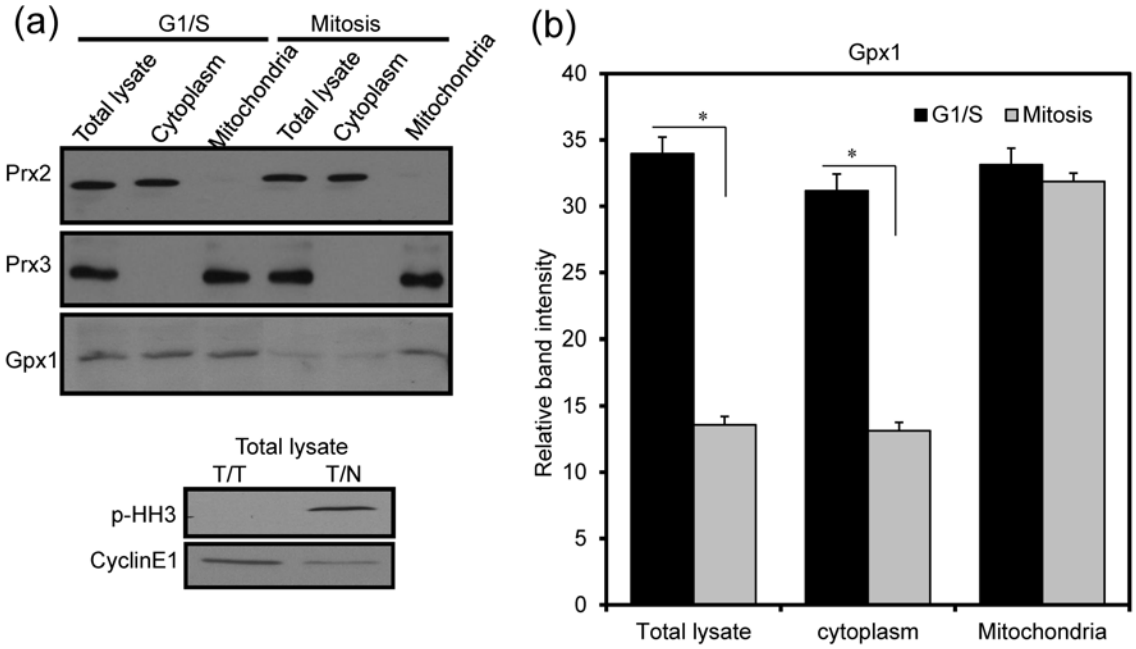
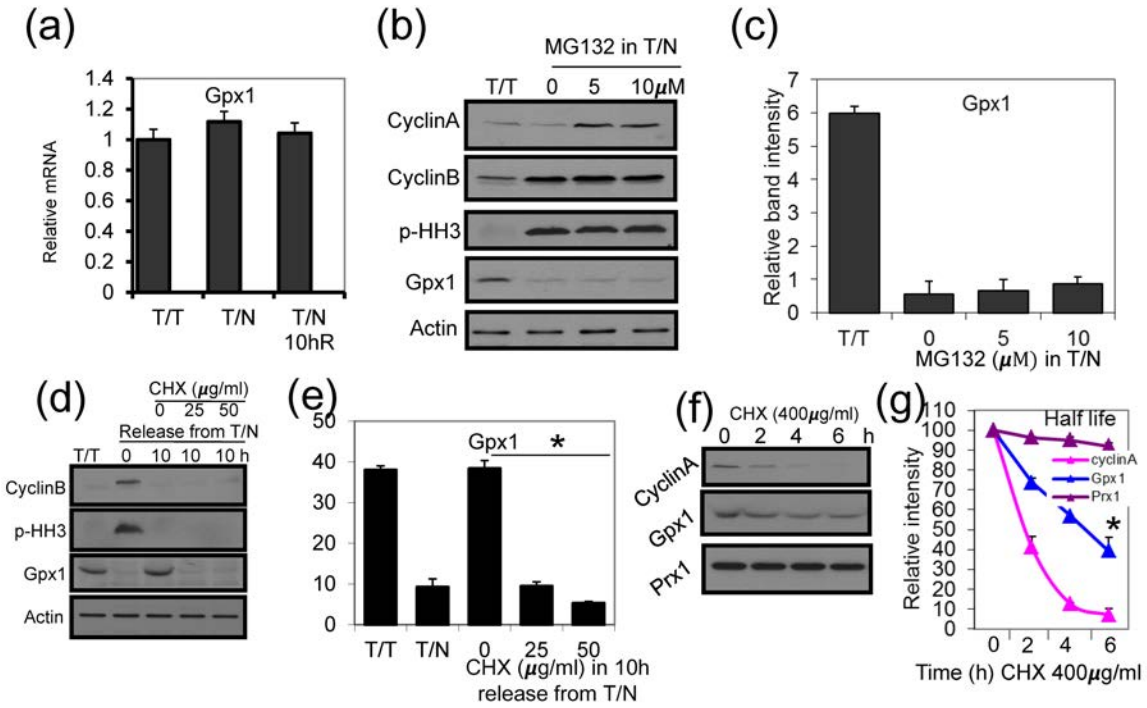

(a)
G1/S
Mitosis
Total lysate
Cytoplasm
Mitochondria
Total lysate
Cytoplasm
Mitochondria
Prx2
Prx3
Gpx1
Total lysate
T/T
T/N
p-HH3
CyclinE1
(b)
Gpx1
G1/S
Mitosis
Relative band intensity
40
35
30
25
20
15
10
5
0
*
*
Total lysate
cytoplasm
Mitochondria

Figure 6


(a)
Gpx1
Relative mRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
T/T
T/N
T/N 10hR
(b)
MG132 in T/N
T/T 0 5 10μM
CyclinA
CyclinB
p-HH3
Gpx1
Actin
(c)
Gpx1
Relative band intensity
7
6
5
4
3
2
1
0
T/T
0
5
10
MG132 (μM) in T/N
(d)
CHX (μg/ml)
0 25 50
Release from T/N
T/T 0 10 10 10 h
CyclinB
p-HH3
Gpx1
Actin
(e)
Gpx1 *
50
40
30
20
10
0
T/T T/N 0 25 50
CHX (μg/ml) in 10h release from T/N
(f)
CHX (400μg/ml)
0 2 4 6 h
CyclinA
Gpx1
Prx1
(g)
Half life
Relative intensity
110
100
90
80
70
60
50
40
30
20
10
0
cyclinA
Gpx1
Prx1
*
0 2 4 6
Time (h) CHX 400μg/ml