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Research Article

Mouse WDR13 is involved in a Novel Ubiquitin-ligase, Activated during Meiosis, Apoptosis and Stress-response

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Abstract

Background: WDR13 is known to function either as an M-phase check point protein or F-box-ubiquitination protein, both involved in regulating the cell cycle. WDR13 participates in poly-ubiquitination mediated protein degradation, contributing to several functions like stress- response and apoptosis. **Methods**: Mouse testis (MT) primary, testis-derived TM3 and mouse embryonic teratocarcinoma PCC4 cells were incubated with TG-β, MG132 and retinoic acid. Silencing WDR13 and inhibition of ubiquitin-ligase complex with MG132 TGF-ß (Transforming Factor Beta) was performed. **Result:** Up regulation of WDR13 upon incubations with TG-β, MG132 and retinoic acid suggests its participation in cell cycle, stress-response and apoptosis. Incubation of testis derived TM3 cells with retinoic acid up regulated WDR13 and meiotic markers, implicating WDR13 in meiosis. Silencing WDR13 and inhibition of ubiquitin-ligase complex with MG132 revealed that WDR13 and Fbw7 compliment the functions of each other. Knocking WDR13 and Fbw7 together enhanced the extent of apoptosis. **Conclusions**: WDR13 apparently participates in a novel ubiquitin-ligase, activated during cell cycle, stress-response and/or apoptosis. When Fbw7 and WDR13 are knocked-down together both participate in ubiquitin-ligases, functionally complimentary to each other, possibly implying obligatory consequence of apoptosis in cells. These results enhance our understanding of the biology of cancer cells.

Keywords: WDR13, poly-ubiquitination , Transforming Factor Beta.

Introduction

Genes localized on the sex chromosomes (X and Y) are expected to contribute in important functions such as spermatogenesis, testicular differentiation /development, sex-determination etc., Singh and co-workers (Suresh et al., 2005) sequenced several chromosomal regions and identified a novel WDR13 gene, localized on mouse X chromosome (mapping to XA1.1 locus) and predominantly expressed in the testis. WDR13 contained 9 exons and 8 introns and is expressed right from early stage of gonadal development, throughout adult life with predominant expression in germ cells of adult testis (Suresh et al., 2005). Alternatively spliced and significantly varied expression of WDR13 transcripts observed in several tissues suggested functional diversity of the gene (Singh et al., 2003). Expression even in unfertilized eggs and neuronal stem cells indicated functional significance of WDR13 in early stages of mouse development (Suresh et al., 2005). Analysis of EST clones from various tissues identified two splice variants: a full-length 485 amino acid (aa) ~ 53-kDa protein and a 393-aa, ~ 43-kDa protein, devoid of N-terminal 92 aa region (Suresh et al.,2005). Significant levels of expression of WDR13 in the early embryos, testis and ovary suggested involvement of WDR13 in differentiation/development/ maintenance of gonads. Proteins forming WD-repeat family are characterized by the presence of repeated sequences of about 44-60 aa with conserved spacing and core, typically bordering GH (glycine-histidine) at the amino and WD

(tryptophan-aspartic acid) carboxy termini of the repeat units and proteins contain 4-16 of such repeat units (Smith et al., 1999). WD-repeat proteins are identified as regulatory components in multi-protein complexes governing several processes such as transcriptional activation/repression, signal transduction, ubiquitination, cell cycle entry/exit, pre-mRNA / rRNA processing, chromatin modification, RNA export, cell death, microtubule assembly and protein transport to/from and across membranes (Smith et al., 1999). Functional sub-families of WD repeat proteins have been identified on the basis of the criterion of surface similarities in the propeller structure, which presumably reflects similarities in binding partners among the members of sub-families (Smith et al.,1999). Upon cloning and over expression, 43 kDa WDR13 was insoluble and underwent autolysis during folding, possibly due to the presence of a large number of unstable peptide bonds (Murthy et al .,2008). Realizing that WDR13 is an unstable protein, we analysed a collection of literature reported WD-repeat proteins for their Protein Instability Index (PII) and observed clustering of these proteins according to PII and function (Murthy et al ., 2008). WDR13 clustered with M-phase checkpoint proteins on one side and F-box proteins associated with E3-ubiquitin-ligase, responsible for protein degradation on the other, both possibly contributing to regulation of cell cycle (Kile et al 2002). Further analyses revealed that WDR13 possessed novel SOCS (Suppressor of Cytokine Signaling) and NLS (Nuclear Localization Sequence), perhaps suggesting involvement of WDR13 in attenuating cytokine signals and localizing into the nucleus. BLAST of WDR13 sequence in the database of interacting proteins revealed that WDR13 is homologous to hCdc20, expressing Fbw7, participating in SCF-ubiquitin-ligases, associated APC/C, involved in protein degradation. To know the in vivo role of Wdr13, we depleted Wdr13 gene expression by ShRNA silencing, interestingly we observed over expression of Fbw7 and activation of apoptotic pathway and also found Wdr13 is activated in specialized cellular condition like cell division(mitosis and meiosis), stress-response and apoptosis. Since bioinformatic aproaches showed Wdr13 is the functional complement of Fbw7 and our experimental results also sugesting possible roles of Wdr13 in protein degradation.

Materials and Methods

Cell Culture and animal maintenance

TM3 and PCC4 cells were obtained from American Type Cell Culture (ATCC) and were grown and maintained in DMEM containing 10% foetal bovine serum albumin in the presence of penicillin (100 U/ml) and streptomycin (50 µg/ml) at 37 °C in a humidified incubator with 5% CO_2 supply and used for subsequent experiments as mentioned below.

Cloning the cDNA transcripts of WDR13

A high-titer CLONETECH mouse testis cDNA library in pACT2 vector was used for PCR amplification of WDR13 transcripts. Full-length (485 aa and 53 kDa) WDR13 was PCR amplified from the library, while 393 aa transcript was amplified from the plasmid containing full-length WDR13. Forward primers used for 485 and 393 aa transcripts were 5'ATTCATATGGCCGCGGTGTGGCAG3' and 5'ATTCATATGCGAATGGAGGACTTTGAA3', respectively, while reverse primer employed for both of them was 5'ATTGAATTCCCCTACTTTTGCTCTCGT3'. Conditions employed in both the PCR reactions were 5 cycles at 48.0º C followed by 25 cycles at 58.0º C. Promega proofreading Taq DNA-polymerase was used in all the PCR reactions for accurate amplification of the cDNAs. Both the 485 and 393 aa cDNAs were cloned between *Nde1* and *EcoR1* restriction sites of pET28 vector. The amplified cDNAs were restriction digested with the respective enzymes, ligated into the vector and resultant plasmids transformed into TOP10 bacteria. Plasmid containing colonies were grown; plasmids purified and transformed into BL21DE3 cells fro protein expression.

Treatment of cells with TGF-ß, MG132, retinoic acid and PCR analysis

Primary cells were prepared from Mouse testis by trypsin and collagenase treatment, employing standard protocols. Mouse testis primary cells; testis-derived TM3 and mouse embryonal teratocarcinoma PCC4 cells established in tissue culture were grown to ~65% confluence were employed in these studies. Cells were incubated for appropriate time at concentrations of TGF-ß - 50 µM, MG132 – 0.5 µM and all Trans retinoic acid - 10 µM and harvested. TGF-ß and

MG132 incubations were performed for a fixed time period of 24 hrs, while retinoic acid treatment was followed as a function of time up to 48 hrs. Total RNA was isolated from cells using the Trizol extraction procedure and to convert mRNA to cDNA using cDNA synthesis kit supplied by Promega Corporation. The cDNA prepared was used for, semi-quantitative PCR reactions. PCR products were analyzed on 1% agarose gel and bands were visualized by staining with ethidium bromide. PCR primers used for common sequences between all transcript of each of WDR13 and Fbw7 (NM_080428) were 5'AAGGGGGGCT-CCAGCAAG3' and 5'ATGATGGC AGGAGGGTTGTTA3' as the forward and 5'CCCTACTTT-TGCTCTCGTCTCC3' and 5'AGCTTGGTTTCCTCAGTTCC3' as reverse primers, respectively. Forward and reverse PCR primers used for β-actin (used as control) were 5'ATCT-GGCACCACACCTTCTACA ATGAG CTC GG3' and 5'CGTCATCCTGCTTG-CTGA TCCA CATC-TGC3' respectively. Primers used for WDR13 and Fbw7 were expected to amplify 570 bp and 565 bp regions, respectively. Semi-quantitative PCR reactions (cycles) were performed using standard conditions at 59.0º C, 51.5º C and 58.0º C, respectively for WDR13, Fbw7 and β-actin; equal amount of template cDNA was taken for each reaction and identical amount of PCR product was used for electrophoresis.

Stress-response study with tissue culture cells and analysis for levels of WDR13

Mouse PCC4 cells were obtained from American Type Cell Culture (ATCC) and were grown to ~65% confluence in tissue culture were subjected to stress-response studies. For heat-shock study, aliquots of 5 million cells were incubated at 42º C for 45 min and then at 37º C for post heat-shock incubation. Aliquots of cells flash frozen at each time point, up to 2 hrs were used for analysis by semi-quantitative PCR. For stress-response study with hydrogen peroxide (H2O2) or MG132, aliquots of 5 million cells were incubated in 0-30 mM H2O2 or 0-2 μM MG132 at 37º C for 2 hrs and aliquots of cells for each H2O2 / MG132 concentration point were taken for PCR analysis. RNA was isolated from cells by Trizol extraction and cDNA was synthesized employing SMART cDNA synthesis kit. Levels of WDR13 and β-actin (used in normalizing the cDNAs) were assessed by semi-quantitative PCR, using primers described earlier.

Primers used for amplifying CHIP (C-terminal HSP-70 interacting protein, a stress-response marker), were 5'ACCAGGAGAGTGAGCTGCAT3' (forward) and 5'GAGCTGTTCCTGGGTCAGAG3' (reverse), PCR of which upon amplification for 25 cycles at 58.0º C, amplified a sequence region of ~370 bp

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Silencing WDR13 and analysis for assessing the levels of their own and those of apoptosis markers in cells:

Sequences of the cDNAs of WDR13 and Fbw7 were subjected to analysis for arriving at the most probable sequences that could effectively silence these genes, using online protocol available at "Gene Link TM and RNAi Explorer TM", available in the internet. ShRNAs were designed employing recommended tools and sense strand of DNA oligos: 5'GACCTTCCAGCCAGTCAACAACAA3' and 5'GTGTGGA-ATGCAGAGACTGGAGA3', for WDR13 and Fbw7, respectively. For gene silencing double stranded DNA oligos were cloned into the *Bbs1* restriction site of mU6Pro vector and plasmids transformed into TOP10 bacteria. Bacterial cultures were grown; ultra-pure plasmid DNA purified using Qiagen Midi-prep DNA purification columns following manufacturers protocols. Purified plasmids were transfected into PCC4 cells employing "Lipofectamine2000" transfection reagent at a concentration of 2.5 μl of reagent + 600 ng of plasmid DNA/10 million cells. Plasmid DNA and transfection reagent were made up with incomplete medium to 250 μl/10 million cells, over layered on the cells and incubated at 37 ° C for 3 hrs. Un-absorbed transfection cocktail was decanted off; cells over layered with adequate volume of complete medium and allowed to grow up to the respective time point. Cells were scrapped from culture plates and quantitatively recovered by centrifugation. Cells were lysed, total RNA isolated, mRNA converted to cDNA and normalized with respect to GAPDH (glyceraldehyde 3-phosphate dehydrogenase). We used semi-quantitative PCR of well-known stress-response markers, TRAIL (TNF (Tumour Necrosis Factor)-Related Apoptosis Inducing Ligand) and Caspase 3, for estimating the extent of apoptosis induced in cells due to silencing WDR13 and/or Fbw7. Primers employed for PCR analysis of TRAIL were 5'CCGGGCAGATCACTACAC3', forward and 5'GACTGAAGGACATGGGGG3', reverse, while those for Caspase 3 were 5'CTCGCT-CTGGTACG

GATG3' forward and 5'GGCAAGCCATCT CC TCAT3', as the reverse primer. Primers employed for amplifying TRAIL and Caspase 3 yielded PCR products of 444 and 366 base pairs, respectively. PCR analyses were performed with identical amount of cDNA templates from various preparations. Intensity of PCR bands were estimated for analyses in methods "g and h" herein employing Singene gel documentation system (Model GGS 12/D-E Gene Genius Classic) and results plotted.

Identical procedures were employed in post-transcriptional silencing WDR13 and/or Fbw7 with ShRNA, cDNA synthesis, semi-quantitative PCR and Confocal microscopy of cells, as described herein.

Results

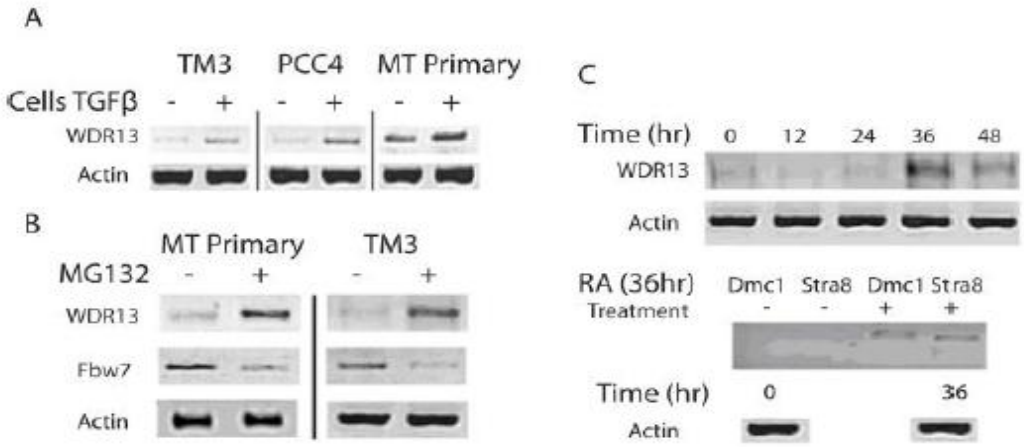
Studies implicating WDR13 in cell cycle processes and meiosis

Incubation of mouse testis (MT) primary, testis-derived TM3 and mouse embryonal teratocarcinoma PCC4 cells with TGF-ß were performed for 24 hrs and analyzed the cells for their levels of WDR13 transcript, by semi-quantitative PCR. Results shown in Fig. 1A reveal that WDR13 is up regulated readily in all the assays, compared to the respective controls. This implied that WDR13 is involved either in signaling and/or regulation of the cell cycle.

Incubation of MT primary and TM3 cells with MG132 were performed for 24 hrs and analyzed the cells for their levels of WDR13 by semi-quantitative PCR. Results shown in Fig. 1B indicated that WDR13 was up regulated readily upon incubation with MG132, compared to the respective controls.

We incubated mouse testis derived TM3 cells with RA as a function of time and looked for changes in the levels of WDR13 and meiosis markers. Very interestingly, results shown in Fig 1C revealed that levels of WDR13 and meiosis markers Dmc1 and Stra8 were up regulated (at 36 hrs) in TM3 cells upon incubation with RA, compared to the controls. This suggests probable involvement of WDR13 in meiosis.

Figure 1: Incubation of tissue culture cells with TGF-ß, MG132 and retinoic acid (RA) at 37º C and analysis by semi-quantitative PCR



A. Incubation of mouse testis (MT) primary, testis derived TM3 and embryonal teratocarcinoma PCC4 cells with 100 µM, concentration of TGF-ß for 24 hrs. Semi-quantitative PCR reactions were performed for WDR13, Fbw7 and β-actin (as control). Results showed an increased amount of WDR13 levels as against Fbw7.

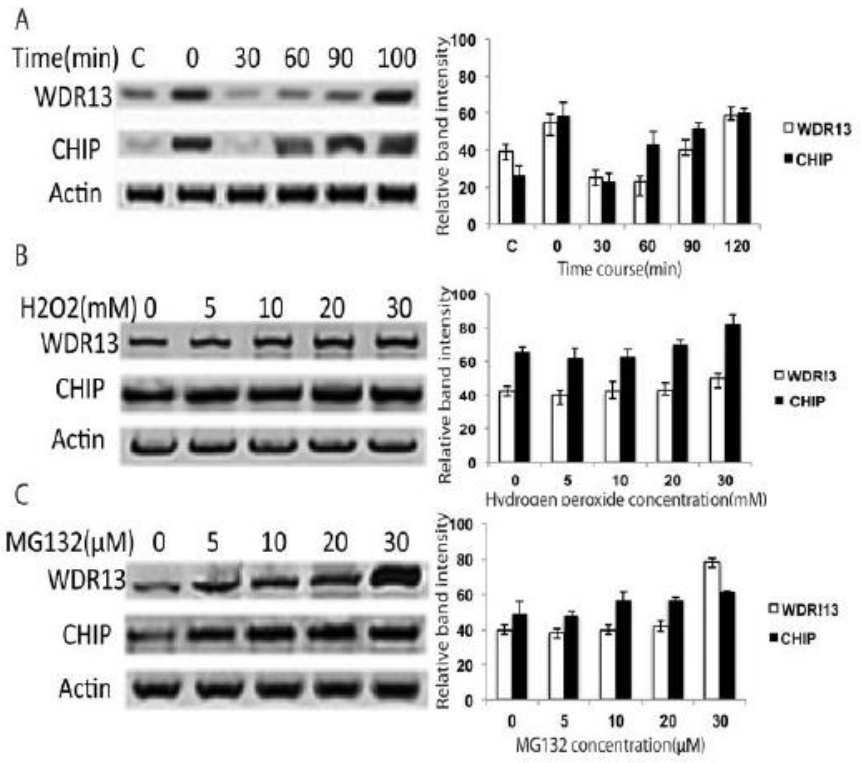
B. Incubation of mouse testis (MT) primary and testis-derived TM3 cells with 100 µM concentration of MG132 for 24. Semi-quantitative PCR reactions were performed for WDR13, Fbw7 and β-actin (as control). Results showed an increased amount of WDR13 levels as against Fbw7.

C. Incubation of mouse testis derived TM3 cells with 100 µM concentrations of RA for 24 hrs. Semi-quantitative PCR reactions were performed for WDR13, meiosis markers: Dmc1, Stra8 and β-actin (as control). Results showed an increased level of WDR13 and meiosis markers Dmc1 and stra8 compared to the controls.

Involvement of WDR13 in stress-response and/or apoptosis

We subjected mouse embryonic teratocarcinoma PCC4 cells to stress by incubation (a) after heat shock (b) with H2O2 (hydrogen peroxide) and (c) with MG132 and cells were analyzed for their levels of various transcripts by semi-quantitative PCR. We employed time course (up to 2 hrs) of incubation of cells subjected to heat-shock and fixed time (2 hrs) of incubation with H2O2/MG132, as a function of concentration. We estimated levels of WDR13, a stress-response marker, CHIP (C-terminal Hsp70-interacting protein) and β-actin, as PCR control. Data of these studies are presented in Fig 2. In all the cases PCR data and bar diagrams of quantified PCR band intensities revealed that WDR13 and CHIP got up regulated gradually during incubation. In the heat-shock study, both WDR13 and CHIP were up regulated immediately upon exposure to heat-shock, while they were also up regulated gradually, during post heat-shock incubation.

Figure 2: Study of stress-response of mouse embryonal teratocarcinoma PCC4 cells in tissue culture and analysis by semi-quantitative PCR.



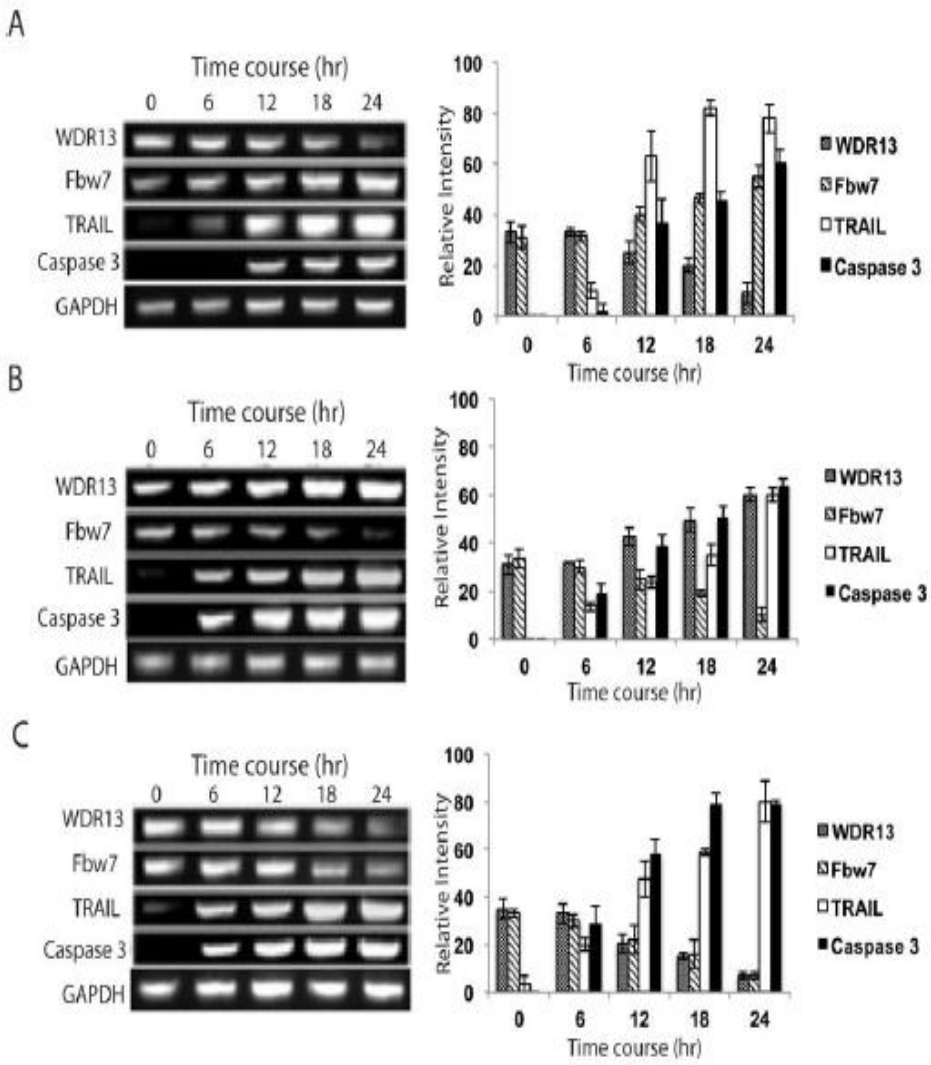
A. Panels a, b and c respectively show agarose gel patterns of the semi-quantitative PCR analyses of WDR13 and CHIP in PCC4 cells with time course of post heat-shock incubation (a) and different concentrations of H2O2 (b) and MG132 (c). In post heat-shock incubation study, 'lane c' represents control sample, not subjected to heat shock, but incubated for the same time (45 min) at 37º C.

Silencing WDR13 and/or Fbw7 employing ShRNA, for assessing Physiological changes in cells

We studied mouse PCC4 cells after post-transcriptional silencing of WDR13 and/or Fbw7 and estimated levels of WDR13 and Fbw7, employing semi-quantitative PCR. Expecting that these conditions cause stress and induce cells into apoptosis, we assessed extent of stress in cells, by estimating the levels of apoptosis markers: TRAIL (TNF (Tumor Necrosis Factor)-Related Apoptosis Inducing Ligand) and Caspase 3, also by semi-quantitative PCR. We also performed confocal microscopy of PCC4 cells subjected to silencing for WDR13 and/or Fbw7 with

ShRNA, which are shown in Fig 3. These results reveal that when WDR13 is silenced, levels of WDR13 decreased, while those of Fbw7 increased gradually (Fig. 3Aa and Ba) as a function of time, clearly showing that Fbw7 is a functional compliment of WDR13. When cells treated with Fbw7 ShRNA (to inhibit SCF-ubiquitin-ligase), levels of Fbw7 decreased, while those of WDR13 increased gradually (Fig. 3Ab and 3Bb), suggesting that WDR13 is a functional compliment of Fbw7. This clearly shows that WDR13 and Fbw7 are functionally complimentary to each other. Interestingly, in both WDR13 silencing (Fig. 3Aa and 3Ba) and Fbw7 silencing (Fig.3Ab and 3Bb), levels of TRAIL and Caspase 3 increased gradually, suggesting that silencing either WDR13 or Fbw7 caused stress in cells and induced cells into apoptosis. Interestingly, when silencing of WDR13 and Fbw7 were combined, levels of WDR13 and Fbw7 decreased gradually, while those of TRAIL and Caspase 3 increased to greater extent (Fig. 3Ac and 3Bc), compared to individually silencing either WDR13 or Fbw7 (Figs. 3Aa & Ba or 3Ab & Bb).

Figure 3: Silencing WDR13 and/or Fbw7 in PCC4 cells employing ShRNA and analysis by semi-quantitative PCR

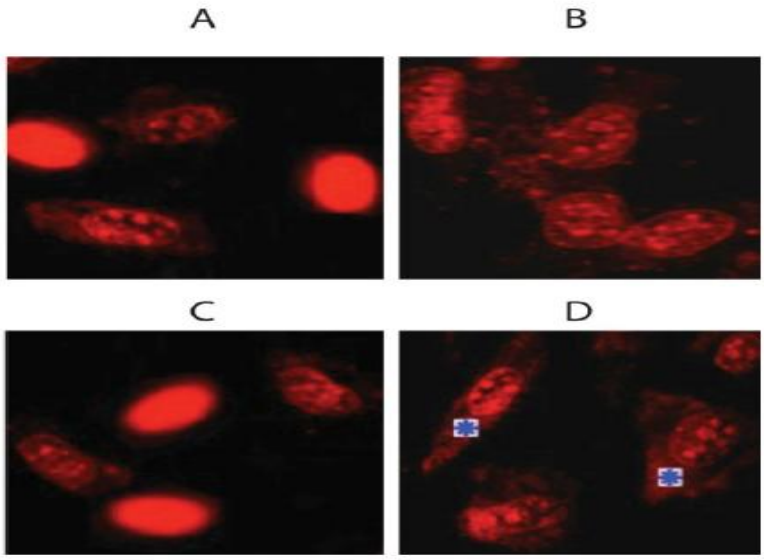


A. Sub sets a, b, and c show amplified PCR bands of "WDR13, Fbw7, TRAIL and Caspase 3", as a function of time, in each case of cells silenced for WDR13 (a), Fbw7 (b), and silenced for WDR13 + Fbw7 (c), by semi-quantitative PCR and PCR product resolved on agarose gel.

Confocal microscopy of PCC4 cells subjected to silencing for WDR13 and/or Fbw7 with ShRNA

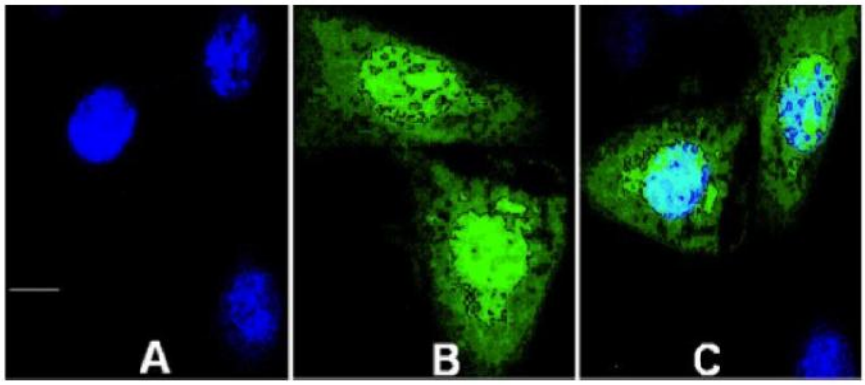
We performed confocal microscopy for monitoring the physiological changes taking place in PCC4 cells using propidium iodide staining after silencing for WDR13 and/or Fbw7 with ShRNA. Results of this study are shown in Fig. 4: PCC4 cells (Fig.4,panel A) Incubation of cells with WDR13 silencing shRNA cause cell cycle arrest and apoptosis (Fig. 4, panel B). When Fbw7 is silencing with ShRNA (Fig. 4, panels C), similar cell cycle arrest and apoptosis were observed. When silencing for WDR13 and Fbw7 were combined, cells showed greater extent of stress and apoptosis, as evidenced by increased levels of TRAIL and Caspase 3 and accumulation of fragmented chromatin into the nucleus, around the nucleolus (Fig.4, panel D).

Figure 4: Confocal Microscopy of PCC4 cells after silencing for WDR13 and/or Fbw7 employing ShRNA



WDR13 and/or Fbw7 were silenced in PCC4 cells employing ShRNA and cells subjected to confocal microscopy, as described in Methods. Panels A, B and C respectively represent pictures of cells silenced for WDR13, Fbw7 and WDR13 + Fbw7, together. Panel D represents Confocal Microscopy of cells silenced for Fbw7, employing ShRNA, as an additional control.

Figure 5: Confocal microscopy of PCC4 cells, expressing GFP-tagged WDR13



Panel "**A**" shows control PCC4 cells stained with DAPI; panel "**B**" shows PCC4 cells expressing GFP-tagged WDR13, while panel "**C**" shows PCC4 cells expressing GFP-tagged 53 kDa WDR13, stained with DAPI.

Discussion

WDR13 was thought to play a role either in testicular differentiation and development or sex-determination, based on it's localization on mammalian X-chromosome and predominant expression in the testicular tissues. Although, only two transcripts are reported earlier, WDR13 is apparently expressed in three isoforms similar to Fbw7 (F-box containing WD-repeat protein, participating in the SCF-Ubiquitin-ligase), as revealed by the BLAST analysis of WDR13 sequence in the database of interacting proteins (4) that WDR13 is homologous to Fbw7. In view of these and earlier functional prediction (4), WDR13 is expected to participate in a novel ubiquitin-ligase, similar to Fbw7.

Considering the fact that proteins involving in E3-ubiquitin-ligase also participate in both signal transduction as well as cell cycle regulation processes (Kile, et al 2002), we studied the effect of incubation of primary and tissue culture cells with TGF-ß (a molecule involved in both these processes) and found that WDR13 gets up regulated readily in the presence of TGF-ß. This confirmed that WDR13 contributed to either any or both of these processes, by participating in an ubiquitin-ligase. Our attempt to inhibit cellular protein degradation with MG132 also resulted in up-regulation of WDR13. Since MG132 inhibits E3-ubiquitin-ligase, results in cell cycle arrest, activating death-receptors leading to apoptosis, up regulation of WDR13 under these conditions implies probable involvement of WDR13 in a novel E3-ubiquitin-ligase (insensitive to MG132), which might compliment the function of Fbw7 by contributing to one or more of these processes. Further, MG132 had also been reported to increase the levels of anti-malignant and anti-metastasis retinoic acid in cells (Andela et al 2004), which also could signal cells into meiosis. Thus, we looked for the effect of incubation of TM3 cells with retinoic acid and noticed up-regulation of both WDR13 and meiosis markers. This result suggests that WDR13 perhaps plays a role in meiosis *in vivo* conditions in the testicular tissue. Since MG132-based inhibition of protein degradation also causes stress in cells, leading to cell cycle arrest, we studied if WDR13 was also involved in stress-response processes in cells. These studies revealed that WDR13 levels upregulated with stress conditions.

Studies implicating WDR13 in 'Ubiquitin-Ligase-mediated' cell cycle processes: TGF-ß (Transforming Factor Beta), a negative regulator of cell division influences a plethora of cellular processes by participating in the signalling pathways of cells (Shi et al., 2003). Bioinformatics analyses identifying a putative SOCS (Suppressor of Cytokine Signalling) region in WDR13 sequence (Murthy et al 2008) indicating apparent involvement of WDR13 in ECS, known to participate in regulation of cell cycle (Vodermaier et al 2004) suggested that WDR13 should respond to presence of TGF-ß in the media of cell cultures. Thus, we incubated mouse testis (MT) primary; testis-derived TM3 and mouse embryonic teratocarcinoma PCC4 cells with TGF-ß for 24 hrs and analyzed the cells for their levels of WDR13 transcript, by semi-quantitative PCR. This also implies that WDR13 is required in cells to counter 'TGF-ß mediated down-regulation' of cell division or necessitated protein degradation and/or apoptosis (Shi et al 2003). MG132, a proteiosome inhibitor of ubiquitin-ligase apparently inhibits protein degradation by caspase-8 activation, causes cell cycle arrest and drives cells into apoptosis (Cervello et al ., 2004). TM3 cells incubated with MG132 also produced apoptotic bodies suggesting that MG132 induced apoptosis in these cells. Up-regulation of WDR13 upon incubation of cells with MG132 suggested involvement of WDR13 in countering the inhibitory effect on ubiquitin-ligase, by contributing to protein degradation or apoptosis, induced by MG132-mediated cell cycle arrest. These results are in agreement with our earlier bioinformatics based postulation (Murthy et al., 2008) regarding involvement of WDR13 in ECS (Elongin B-C-Cullin-SOCS)-ubiquitin-ligase. The precise time of RA accumulation and regulation of RA levels during foetal gonad development provided the molecular switch that specified germ cell fate (Koubova et al 2006; Bowles et al.,2006). We expected that enhancing RA levels in cells derived from testicular tissue might provide a clue for possible involvement of WDR13 in meiosis. Bowles and co-workers (Bowles et al ., 2006) reported that incubation of male urogenital ridge organ cultures with RA induced expression of meiosis markers, suggesting that RA incubation accumulated RA in cells and signalled them into meiosis. Thus, we incubated mouse testis derived TM3 cells with RA as a function of time and looked for changes in the levels of WDR13 and meiosis markers. Levels of WDR13 and meiosis markers Dmc1 and Stra8 were up regulated (at 36 hrs) upon incubation of TM3 cells with RA, compared to

the controls. This suggests probable involvement of WDR13 in meiosis. Our results suggest that WDR13 perhaps plays a role in meiosis in the testicular tissue. Ubiquitin-ligase mediated protein degradation playing an affirmative role in cell division, and meiosis, indeed being a specialized type of cell division, suggests that possible role of WDR13 in protein degradation.

As WDR13 is homologous to Fbw7 (Murthy et al ., 2008) we expected that it might perform a protein degradation function by participating in a novel ubiquitin-ligase, particularly in specialized cellular conditions like cells division (mitosis and meiosis), stress-response and apoptosis. MG132 is known to cause stress in cells by inhibiting protein degradation, necessary for cell division, which activates death-receptors, leading cells into apoptosis. Mouse embryonal teratocarcinoma PCC4 cells were subjected to stress by incubation after heat shock, with H2O2 (hydrogen peroxide) and with MG132 and cells were analyzed for levels of various stress response markers and other transcripts by semi-quantitative PCR. Upon heat shock, levels of WDR13 and CHIP increased (compared to the controls) as expected, but during post heat shock incubation, particularly at lower time points, levels of WDR13 and CHIP appear to be low. This could be due to the relief experienced by cells by shifting from 42° to 37° C. However, when cells assess the damage due to heat-shock and enter apoptotic phase, levels of WDR13 and CHIP get up regulated readily. These results clearly show that WDR13 is a stress-response protein and it could be involved in degrading proteins, denatured during cellular stress.

Silencing WDR13 and/or Fbw7 either by ShRNA, for assessing physiological change in cells: Mouse PCC4 cells were analysed for their levels of WDR13 and Fbw7 by semi-quantitative PCR after post-transcriptional silencing of WDR13 and Fbw7. Expecting that these conditions cause stress and induce cells into apoptosis, for assessing the extent of stress in cells, we also estimated levels of apoptosis markers, TRAIL (TNF (Tumour Necrosis Factor)-Related Apoptosis Inducing Ligand) and Caspase 3 by semi-quantitative PCR.

Confocal microscopy of PCC4 cells subjected to silencing for WDR13 and Fbw7 were also performed. Results reveal that when WDR13 is silenced, levels of WDR13 decreased, while those of Fbw7 increased gradually, as a function of time, clearly showing that Fbw7 is a functional compliment of WDR13. When cells were incubated with MG132 (to inhibit Fbw7 containing SCF-ubiquitin-ligase), levels of Fbw7 decreased, while those of WDR13 increased gradually, suggesting that WDR13 is a functional compliment of Fbw7. This clearly shows that WDR13 and Fbw7 are functionally complimentary to each other. Interestingly, in both WDR13 and Fbw7 silencing, levels of TRAIL and Caspase 3 increased gradually, suggesting that silencing either for WDR13 or Fbw7 caused stress in cells and induced them into apoptosis. When, silencing WDR13 and Fbw7 were combined, levels of WDR13 and Fbw7 decreased gradually, while those of TRAIL and Caspase 3 increased to greater extent, compared to individually silencing either for WDR13 or Fbw7. Earlier, SCF ubiquitin-ligase and APC/C (Anaphase Promoting Complex/Cyclosome) were reported to regulate levels of each other and together the cell cycle (Vodermaier 2004). Levels of apoptosis markers TRAIL and Caspase 3 have gone up even before WDR13 and Fbw7 were silenced either independently, or even when they were combined. This is because when WDR13 and/or Fbw7 are silenced, levels of TRAIL normally go up due to stress in cells and thus levels of TRAIL need not remain un-detectable till levels of WDR13 and/or Fbw7 disappear completely. Further, we considered 10 times higher amount of cDNA template for PCR amplification of Caspase 3. The results clearly suggest that the extent of apoptosis increased to greater extents in cells, when WDR13 and Fbw7 were inactivated together, compared to silencing either WDR13 or Fbw7 independently. These results not only concur, with the earlier report, but also validate the “ELM analyses-based prediction” that “WDR13 participates in complex formation with ECS ubiquitin-ligase, associated with APC/C”. Thus, we conclude that WDR13 is a component of ECS (similar to Fbw7, participating in SCF); ECS and SCF associate with APC/C and constitute alternate routes of protein degradation (see below) during cellular processes like anaphase $\rightarrow$ metaphase transition during cell division, stress-response and apoptosis.

Eukaryotic cells divide by an oscillatory mechanism, progressing by phosphorylation of “cell division control” proteins (Cdcs and Cdks) on one hand and their ubiquitination-mediated proteolysis on the other, involving two major classes of ubiquitin-ligases, SCF

and ECS, both participating in the proteasome, APC/C (Kile et al 2002: Vodermaier 2004 and Meusser et al ., 2005). Inhibition of ubiquitination-mediated protein degradation with ubiquitin-ligase inhibitors would accumulate spent Cdc and Cdk molecules and cause hindrance in cell division, leading to cell cycle arrest and apoptosis. In view of this, inhibition of ubiquitination employing peptide inhibitors is employed as an approach for arresting tumour cell division, thereby inducing cells into apoptosis. Designing molecules specifically inhibiting all the ubiquitin-ligase (SCF or ECS) complexes has been a difficult task as all of such complexes are not known, components of these complexes also perform non-proteolytic (ubiquitination) functions, all of which are not fully understood ((Kile et al 2002: Vodermaier 2004 and Chen 2005) and molecules like MG132 designed for blocking protein degradation also possess biological properties, other than specific inhibition of SCF-ubiquitin-ligase. Thus identification of true and functionally complementary ubiquitin-ligases like Fbw7 and WDR13, shall lead to investigation of their specific inhibitors that would efficiently block ubiquitination, an obligatory step in degrading the cell cycle regulatory proteins. Fbw7 inactivating mutations have been found both in cancer cell lines (Koepp et al .,2001: Moberg et al ., and Strohmaier et al 2001) and primary cancers (Spruck et al ., 2002: Ekholm-Reed et al ., 2004 and . Rajagopalan et al 2004), and Fbw7 loss in cultured cells caused genetic instability (Rajagopalan et al 2004). Knock down of Fbw7 lead to enlarged cells/nuclei (Welcker et al., 2004), polyploidy, multiple centrosomes, reduced cell division and apoptosis (Nakayama et al., 2000). Thus, loss of Fbw7 function leads to profound effect on cell survival, although cells still survive (Nakayama et al ., 2000). This is, perhaps, possible as Fbw7 containing SCF and WDR13 containing ECS control each other and together (both) regulate the cells cycle (Vodermaier 2004) and WDR13 perhaps complements the vital functions of Fbw7 during inactivation of Fbw7 and facilitates survival of cells, although the ability for regulation of cell division is lost. Expression of human WDR13 in several tumour tissues (see compiled expression information available at the database: Hs. 12142) perhaps testifies this phenomenon. Further, Fbw7 containing SCF plays a major role in degrading cyclin E and p27kip during S _ G2 and G2 _ M, while ECS containing proteasome complexes catalyzes degradation of securin and other substrates during $G1 \rightarrow S$ and $M \rightarrow G1$ phases (Vodermaier 2004 and Nakayama et al ., 2000).). In view of the foregoing, such a cross talk of function between analogous proteasome complexes is very much expected, as their common objective is perhaps survival of the cell.

Administering MG132 to actively dividing cancer cells does not produce complete cell cycle arrest as MG132 appears to inhibit not only the SCF complex, but also involve in non-proteolytic (ubiquitination) functions involving NF-kB mediated signaling processes and activation of caspases, ß-catenin, death receptor 5, which induce cells into apoptosis (Andela et al ., 2004 ;Vodermaier 2004 ;Cervello et al ., 2004 ; Chen 2005 and Yoshida et al ., 2005) In view of this, MG132 perhaps induces apoptosis in cells more due to up-regulation of caspases, ß-catenin and death-receptor 5, because cells exposed to MG132 apparently up-regulate ECS (perhaps involving WDR13) for meeting the protein degradation demand (results herein), which is an alternative route of protein degradation. Thus, we believe that developing and administering specific inhibitors that block both SCF and ECS complexes would completely inhibit ubiquitination-mediated protein degradation and lead to cell cycle arrest, thereby inducing cells into apoptosis. As Fbw7 and WDR13 are functionally complementary, gene knock down of either of them in embryos may not be lethal to embryos, while knocking them together might prove to be lethal. Genetic mutations causing loss of Fbw7 function in several human tumour cell lines (Koepp et al., 2001; Moberg et al., and Strohmaier et al 2001), primary cancers (Spruck et al., 2002; . Ekholm-Reed et al., 2004; and Rajagopalan et al., 2004) and cultured cells (Rajagopalan et al .,2004) support the above argument. Gene knock out of WDR13 produced normal mouse embryos (Singh et al.,2008), suggesting complementation of WDR13 function in these animals by an alternative mechanism. Further, development and application of inhibitors to all of APC/CCdc4, APC/CWdr13, APC/CCdc20 and APC/CCdh1, which are involved in protein degradation at different stages of mitotic cell division (Vodermaier 2004) together, shall prove valuable for achieving the desired cell cycle arrest in tumour cells, as mutations in some of these could still lead to unsuccessful cell cycle arrest when an inhibitor of one of these ubiquitin-ligase alone is administered.

Conclusion

The data presented suggests that WDR13 participates in protein degradation, inhibition of which together with Fbw7 would potentially lead to cell cycle arrest and apoptosis in tumour cells.

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