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**Inhibition of ERN1 modifies the hypoxic regulation of the expression of TP53-related genes in U87 glioma cells**

**Abstract:** Inhibition of ERN1 (endoplasmic reticulum to nuclei 1), the major signalling pathway of endoplasmic reticulum stress, significantly decreases tumor growth. We have studied the expression of tumor protein 53 (TP53)-related genes such as TOPORS (topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase), TP53BP1 (TP53 binding protein 1), TP53BP2, SESN1 (sestrin 1), NME6 (non-metastatic cells 6), and ZMAT3 (zinc finger, Matrin-type 3) in glioma cells expressing dominant-negative ERN1 under baseline and hypoxic conditions. We demonstrated that inhibition of ERN1 function in U87 glioma cells resulted in increased expression of RYBP, TP53BP2, and SESN1 genes, but decreased expression of TP53BP1, TOPORS, NME6, and ZMAT3 genes. Moreover, inhibition of ERN1 affected hypoxia-mediated changes in expression of TP53-related genes and their magnitude. Indeed, hypoxia has no effect on expression of TP53BP1 and SESN1 in control cells, while resulted in increased expression of these genes in cells with inhibited ERN1 function. Magnitude of hypoxia-mediated changes in expression levels of RYBP and TP53BP2 was gene specific and more robust in the case of TP53BP2. Hypoxia-mediated decrease in expression levels of TOPORS was more prominent if ERN1 was inhibited. Present study demonstrates that fine-tuning of the expression of TP53-associated genes depends upon endoplasmic reticulum stress signaling under normal and hypoxic conditions. Inhibition of ERN1 branch of endoplasmic reticulum stress response correlates with deregulation of p53 signaling and slower tumor growth.

**Keywords:** endoplasmic reticulum stress, IRE1, ERN1, RYBP, TP53BP1, TP53BP2, TOPORS, SESN1, ZMAT3, NME6, hypoxia.

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endoribonuclease activity is involved in the degradation of a specific subset of mRNA and also initiates the cytosolic splicing of the pre-XBP1 (X-box binding protein 1) mRNA whose mature transcript encodes a transcription factor that stimulates the expression of unfolded protein response specific genes [3,8].

The TP53 protein is one of the best-studied tumor suppressors, which responds to diverse cellular stresses to regulate expression of its target genes, thereby inducing cell cycle arrest, apoptosis and senescence [9,10]. Activation of TP53 begins through a number of mechanisms including phosphorylation by different protein kinases as well as methylation and acetylation [11]. Phosphorylation of the amino terminus of TP53 leads to a conformational change that prevents MDM2 (TP53 E3 ubiquitin protein ligase homolog) binding, resulting in TP53 stabilization and allows it increased interaction and acetylation by CBP/p300, which in-turn enhances TP53 transcriptional activity.

Stability as well as activity of TP53 depends upon different factors. For example, death effector domain-associated factor RYBP (RING1 and YY1-binding protein) also known as YEAF (YY1 and E4TF1-associated factor 1) and DADAF (DAD-associated factor) inhibits ubiquitination and subsequent degradation of TP53, and thereby plays a role in regulating transcription of TP53 target genes. This factor interacts with MDM2 and decreases MDM2-mediated TP53 ubiquitination, stabilizing TP53 and increasing its activity [12]. RYBP is decreased in human cancer tissues and is implicated in the regulation of the transcription as a repressor of the transcriptional activity of E4TF1 and promotes apoptosis [12,13].

Recent data indicates that TP53 binding proteins (TP53BP1, TP53BP2, and TP53BP3) modulate TP53 function, suppress tumor growth, and promote susceptibility to apoptosis, but their activity depends upon different factors [14-19]. Thus, TP53BP1 has a role in checkpoint signaling during mitosis, enhances TP53-mediated transcriptional activation and plays a role in the response to DNA damage [20,21]. TP53BP2 is a member of the ASPP (apoptosis-stimulating protein of p53) family of TP53 interacting proteins which are down regulated in tumor tissues and is required for the induction of apoptosis by TP53-family proteins [19]. Moreover, TP53BP2 promotes DNA binding and transactivation of TP53-family proteins on the promoters of pro-apoptotic genes. TP53BP3, also known as SUMO1-protein E3 ligase TOPORS, regulates TP53 stability through ubiquitin-dependent degradation and is involved in cell growth, cell proliferation and apoptosis [18,22,23].

Non-metastatic cells 6 (NME6), also known as NME/NM23 nucleoside diphosphate kinase 6, participates in oncogenesis and inhibits TP53-induced apoptosis [24,25]. SESN1 (sestrin 1 or TP53 regulated PA26 nuclear protein) and ZMAT3 (Zinc finger, Matrin-type 3 or TP53 target zinc finger protein) are TP53 target genes which have a role in the TP53-dependent growth regulatory pathway [26,27]. ZMAT3 is an mRNA stability-regulating protein which prevents cellular senescence by regulating p21 mRNA decay through control of RISC recruitment [27]. It contributes to TP53-mediated apoptosis by regulation of TP53 expression and translocation to the nucleus and nucleolus. Moreover, ZMAT3 knockdown causes a dramatic inhibition of N-MYC expression and triggers differentiation in neuroblastoma cells [28,29].

The aim of this study was to investigate the effects of hypoxia on the expression of TP53-related genes (RYBP, TP53BP1, TP53BP2, TOPORS, SESN1, ZMAT3, and NME6), those protein products participate in the regulation of cell proliferation and apoptosis in glioma cells, and to study the contribution of endoplasmic reticulum stress sensor ERN1, to fine tune their expression.

## 2 Material and Methods

**Reagents.** The glioma cell line U87 was obtained from ATCC (USA) and grown in high glucose (4.5 g/l) Dulbecco’s modified Eagle’s minimum essential medium (DMEM; Gibco, Invitrogen, USA) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio, Inc., USA), penicillin (100 units/ml; Gibco, USA) and streptomycin (0.1 mg/ml; Gibco) at 37°C in a 5% CO2 incubator.

**Cell lines.** In this work we used two sublines of this glioma cell line. One subline was obtained by selection of stable transfected clones with overexpression of vector (pcDNA3.1), which was used for creation of dominant-negative constructs (dnERN1). This untreated subline of glioma cells (control glioma cells) was used as control 1 in the study of the effects of hypoxia on the expression level of TP53, RYBP, TP53BP1, TP53BP2, TOPORS, SESN1, ZMAT3, and NME6 genes (Table 1). Second subline was obtained by selection of stable transfected clones with overexpression of dnERN1 and has suppressed both protein kinase and endoribonuclease activities of this bifunctional signaling enzyme of endoplasmic reticulum stress. These cells were a gift from prof. M. Moenner (France) [4]. The expression level of the studied genes in these cells was compared with cells, transfected by vector (control 1). Subline which overexpresses dnERN1 was also used, as control 2, for the investigation of the effect of hypoxic condition on the expression level of the above genes under ERN1 enzyme function blockade.
Hypoxia induction. For creation of hypoxic conditions, the culture plates were exposed in a special incubator with 3% oxygen, 5% CO₂, and 92% nitrogen mix for 16 hrs.

Proliferation assay. The proliferation rate of control glioma cells and ERN1 knockdown cells were measured by using a cell counter (Coultronics, Margency, France). Cell number was measured in triplicates after 3 days.

RNA isolation. Total RNA was extracted from glioma cells using Trizol reagent according to manufacturer protocols (Invitrogen, USA). The RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water. For additional purification RNA samples were re-precipitated with 95% ethanol and re-dissolved again in nuclease-free water.

Reverse transcription and qPCR analysis. QuantiTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis according to manufacturer protocol. The expression level of RYBP, TP53BP1, TP53BP2, TOPORS, SESN1, ZMAT3, NME6, and ACTB mRNA were measured in glioma cell line U87 and its subline (clone 1C5) by real-time quantitative polymerase chain reaction using Mx 3000P QPCR (Stratagene, USA) and Absolute qPCR SYBRGreen Mix (Thermo Fisher Scientific, Abgene House, UK). Polymerase chain reaction was performed in triplicate using specific primers which were received from Sigma-Aldrich (USA).

An analysis of quantitative PCR was performed using special computer program Differential Expression Calculator. The values of the expression of TP53, RYBP, TP53BP1, TP53BP2, TOPORS, SESN1, ZMAT3, NME6, and ACTB mRNA were normalized to beta-actin mRNA expressions and represent as percent of control 1 (100%). Statistical analysis was performed according to Student’s test using OriginPro 7.5 software. All values are expressed as mean ± SEM from triplicate measurements performed in 4 independent experiments.

3 Results

3.1 ERN1 modulates expression of TP53-related genes in un-stressed cells

To test the effect of ERN1 on expression levels of TP53-related genes, we used the U87 glioma cells line, which constitutively expresses dominant-negative mutant of ERN1. Expression of this dnERN1 mutant was shown to have an inhibitory effect on ribonuclease and kinase activity of endogenous ERN1 [30]. Figure 1 demonstrates that inhibition of ERN1 gene function in U87 glioma cells affects the expression of several TP53-related genes, with more robust effect on RYBP, Table 1.

**Table 1.** Characteristics of TP53-related genes and primers used for qPCR.

| Gene symbol | Gene name                                                                 | Primer’s sequence | Nucleotide numbers in sequence | GenBank accession number |
|-------------|---------------------------------------------------------------------------|-------------------|--------------------------------|-------------------------|
| TP53        | Tumor protein 53                                                          | F: 5’- ggcccacttcacggtactaa R: 3’- gggtttaaaggccagagt | 1694–1713 1849–1830      | NM_000546               |
| RYBP        | (YEAF, DADAF) RING1 and YY1-binding protein; ring1 interactor (YY1 and E4F1-associated factor 1; DAD-associated factor) | F: 5’- tgacattcagctgtggcttt R: 3’- tccagccttttctctgt | 1944–1963 2221–2202      | NM_012234               |
| TP53BP1     | Tumor protein 53 binding protein 1                                         | F: 5’- cagtcctccaagacgacttg R: 3’- cgggaacagagaaaaagcag | 3229–3248 3463–3444      | NM_005657               |
| TP53BP2     | (ASPP2) Tumor protein 53 binding protein 2 (apoptosis-stimulating of TP53 protein 2) | F: 5’- aggttctgacacggaagcttg R: 3’- cagtgggagttgtgtttctt | 2345–2364 2592–2573      | NM_005426               |
| TOPORS      | Topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase (tumor protein 53 binding protein 3) | F: 5’- ttctctacggtgaggttctg R: 3’- tgtacgctcctggttcg | 566–585 814–795          | NM_005802               |
| NME6 (NME/ NMM2) | Non-metastatic cells 6 (nucleoside diphosphate kinase 6) | F: 5’- tctatacgtggaggtttgctg R: 3’- taggtccctggtctcacta | 409–428 619–600          | NM_005793               |
| SESN1       | Sestrin 1 (TP53-activated gene 26; TP53 regulated PA26 nuclear protein)   | F: 5’- gcatgttcaacattttcttg R: 3’- gttctttgctcctg | 1641–1660 1830–1811      | NM_014454               |
| ZMAT3       | Zinc finger, Matrin-type 3 (TP53-activated gene 608 protein; TP53 target zinc finger protein) | F: 5’- gaattccggcagcatttaga R: 3’- aagttctctcaccaccacccc | 1641–1660 1830–1811      | NM_006212               |
| ACTB        | beta-actin                                                               | F: 5’- ggacattcagacgagatgg R: 3’- aagttctgtgctgctagag | 747–766 980–961          | NM_001101               |
TOPORS, and SESN1 genes, where the expression of RYBP, TP53BP2, and SESN1 genes is increased in glioma cells at this experimental condition. While, the expression of TOPORS, NME6, TP53BP1, and ZMAT3 genes are decreased in glioma cells expressing dnERN1. Therefore, the inhibition of ERN1 gene function in U87 glioma cells modulates the expression of studied TP53-related genes, affecting growth regulation, lowering DNA-damage repair capacity and potentially modulating death signaling.

3.2 ERN1 modifies expression levels of TP53 under hypoxic conditions.

To test whether ERN1 modulates expression of TP53 during hypoxia, control and cells harboring dnERN1 were cultured at normal and low levels of oxygen and expression of TP53 was analyzed by qPCR. As shown in Figure 2A, the level of TP53 mRNA is significantly increased (more than 2 fold) in glioma cells expressing dnERN1. Control glioma cells (stable transfected by vector) show slight decrease in the levels of TP53 mRNA (-14 %) under hypoxic condition. No significant changes were found under hypoxic condition in dnERN1 glioma cells (stable transfected by dnERN1). The changes in expression levels of TP53 mRNA in control and dnERN1 glioma cells seen at the different conditions described above correlate with TP53 protein levels (Figure 2B). Thus, ERN1 modifies the effect of hypoxia on the expression of TP53 gene in U87 glioma cells, where cells with low ERN1 activity will have higher levels of TP53.

3.3 ERN1 modulates expression of the subset of TP53-related genes under hypoxic conditions.

We next tested whether ERN1 also participates in regulation of TP53-related genes during hypoxia. We found that expression levels of RYBP, protein that regulates TP53 stability, are significantly increased under hypoxic condition in both control glioma cells (stable transfected by vector) and in cells expressing dnERN1 (stably transfected with dnERN1) (Figure 3). We therefore concluded that, ERN1 down-regulation has no effect on hypoxic regulation of RYBP gene expression in glioma cells. We found that hypoxia-induced down-regulation of NME6, gene important for DNA metabolism and inhibition of TP53-induced apoptosis, is also not significantly affected by dnERN1 (Figure 4).

mRNA levels of TOPORS, another regulator of TP53 stability, are decreased during hypoxia in both control and dnERN1 glioma cells, but the down-regulation of TOPOS expression was more profound in cells harboring dnERN1 (Figure 5).

Figure 1: The expression of RYBP, TP53BP1, TP53BP, NME6, SESN1 and ZMAT3 mRNA in glioma cell line U87, transfected with vector, and its subline with knockdown of the signaling enzyme ERN1 (dnERN1) measured by qPCR. Values of these mRNA expressions were normalized to beta-actin mRNA and represent as percent of control (100 %); mean ± SEM; n = 4; * – P < 0.05 as compared to control.

Figure 2: Expression of TP53 (tumor protein 53) mRNA (A; qPCR) and protein (B; Western immunobloting) in glioma cell line U87 (Vector) and its subline with a deficiency of the signaling enzyme ERN1 (dnERN1): effect of hypoxia (3 % oxygen, 16 hrs). Values of TP53 mRNA expressions were normalized to beta-actin mRNA expression and represent as percent of control 1 (100 %); mean ± SEM; n = 6; * – P < 0.05 as compared to control 1. Ctrl – control; Hx – hypoxia. N – Relative protein TP53 intensity normalized to actin.

Analysis of mRNA of TP53BP1 and TP53BP2, genes that modulate TP53 function, has shown that hypoxia does not affect TP53BP1 expression in control cells in
statistically significant manner, while cells harboring dnERN1 show slight but statistically significant increase in TP53BP1 during hypoxia (Figure 6). Expression of TP53BP2 was induced during hypoxia in both control and dnERN1 glioma cells, induction was much more robust in cells expressing dnERN1 (Figure 7).

Levels of mRNA of two regulators of TP53 action, SESN1 and ZMAT3, which participate in regulation of cell growth and differentiation, were analyzed as well. The expression of SESN1 is not affected by hypoxia in control glioma cells. Inhibition of ERN1 resulted in up-regulation of SESN1 by hypoxia (Figure 8). mRNA levels of ZMAT3 are decreased during hypoxia in control glioma cells. This decrease was lost in dnERN1 glioma cells (Figure 9). Thus, the inhibition of ERN1 function during hypoxia in U87 glioma cells has suppressive effect on only two of analyzed p53 targets. In conclusion, we demonstrated that ERN1 participates in fine-tuning of mRNA levels of the subset of genes important for TP53 stability and function.

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**Figure 3:** Expression of RYBP (RING1 and YY1-binding protein; YY1 and E4TF1 associated factor 1) mRNA in glioma cell line U87 (Vector) and its subline with a deficiency of the signaling enzyme ERN1 (dnERN1): effect of hypoxia. Values of RYBP mRNA expressions were normalized to beta-actin mRNA expression and represent as percent of control (100%); mean ± SEM; n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2.

**Figure 4:** Non-Metastatic Cells 6 (NME6; NME/NM23 Nucleoside Diphosphate Kinase 6) mRNA expression in glioma cell line U87 (Vector) and its subline with knockdown of the signaling enzyme ERN1 (dnERN1): effect of hypoxia. Values of NME6 mRNA expressions were normalized to beta-actin mRNA expression and represent as percent of control (100%); mean ± SEM; n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2.

**Figure 5:** Expression of TOPORS (topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase; tumor suppressor TP53 binding protein 3) mRNA in glioma cell line U87 (Vector) and its subline with a deficiency of the signaling enzyme ERN1 (dnERN1): effect of hypoxia. Values of RYBP mRNA expressions were normalized to beta-actin mRNA expression and represent as percent of control (100%); mean ± SEM; n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2.

**Figure 6:** TP53 binding protein 1 (TP53BP1) mRNA expression in glioma cell line U87 (Vector) and its subline with knockdown of the signaling enzyme ERN1 (dnERN1): effect of hypoxia. Values of PERP mRNA expressions were normalized to beta-actin mRNA expression and represent as percent of control (100%); mean ± SEM; n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2.
3.4 Inhibition of ERN1 suppresses tumor growth

We showed that inhibition of ERN1 results in changes in expression of different TP53-related genes. To see whether those changes affect cell proliferation, cells with and without dnERN1 were grown under normal conditions. Figure 10 demonstrates that the proliferation rate of cells expressing dnERN1 is two fold lower after 3 days in culture as compared to control U87 glioma cells. Therefore, inhibition of ERN1 can result in suppression of malignant proliferation possibly though deregulation of TP53 signaling.

**Figure 7:** Hypoxic regulation of TP53 binding protein 2 (TP53BP2) mRNA expression in glioma cell line U87 (Vector) and its subline with blockade of ERN1 (dnERN1). Values of TP53BP2 mRNA expressions were normalized to beta-actin mRNA expression and represent as percent of control (100 %); mean ± SEM; n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2.

**Figure 8:** Expression of SESN1 (sestrin 1; TP53 regulated PA26 nuclear protein) mRNA in glioma cell line U87 (Vector) and its subline with a deficiency of the signaling enzyme ERN1 (dnERN1): effect of hypoxia. Values of SESN1 mRNA expressions were normalized to beta-actin mRNA expression and represent as percent of control (100 %); mean ± SEM; n = 4; * – P < 0.05 as compared to control 1; ** – P < 0.05 as compared to control 2.

**Figure 9:** Expression of ZMAT3 (zinc finger, Matrin-type 3; TP53-activated gene 608 protein) mRNA in glioma cell line U87 (Vector) and its subline with a deficiency of the signaling enzyme ERN1 (dnERN1): effect of hypoxia (3 % oxygen, 16 hrs). Values of ZMAT3 mRNA expressions were normalized to beta-actin mRNA expression and represent as percent of control (100 %); mean ± SEM; n = 4; * – P < 0.05 as compared to control 1.

**Figure 10:** The proliferation rate of control glioma cells (Vector) and ERN1 knockdown cells (dnERN1). Cell number was measured in triplicates after 3 days by using a cell counter (Coultronics, Margency, France) and represent as percent of control (100 %); mean ± SEM; n = 4; * – P < 0.05 as compared to control 1.

4 Discussion

In this study we have shown that the inhibition of the ERN1 gene function in U87 glioma cells increases both the level of mRNA and protein of TP53 (Fig. 2), which coordinates diverse cellular functions through the regulation of the expression of target genes, thereby inducing cell cycle arrest and apoptosis [9,31]. We have also demonstrated that ERN1 inhibition changes the expression of several TP53-related genes. Expression of *RYBP*, *TP53BP2*, and
SESN1 genes, which enhance TP53 stability and function, is elevated in glioma cells when ERN1 function is inhibited (Fig 1). This increase may contribute to the higher levels and activity of TP53, which in turn may lead to the suppression of cell proliferation and growth of these cells [4,5,12,13]. Indeed, increased expression of TP53 and RYBP genes in cells with reduced ERN1 activity were associated with suppression of cell proliferation and glioma growth (Fig 10) [4,5] and correlates well with a reported decrease in RYBP expression in multiple human cancer tissues [13]. Increased expression of both TP53 and SESN1 can mediate inhibition of cell growth via activating of AMP-activated protein kinase by TP53 [34]. While higher levels of TP53BP2 mRNA (Fig. 7 or Fig 1), a member of the ASPP family of TP53 interacting proteins which are required for the induction of apoptosis by TP53-family proteins and are down regulated in many human malignancies [19], could also contribute to the suppression of glioma cells proliferation upon ERN1 inhibition.

We have demonstrated that the expression of NME6, TOPORS, and ZMAT3 genes is decreased in glioma cells with inhibited ERN1 (Fig. 1). This decrease may also contribute to the suppression of cell proliferation and tumor growth [4,5], because NME6 is an inhibitor of TP53-induced apoptosis [35,36]. TOPORS is an E3 ubiquitin protein ligase that regulate TP53 stability [37], and ZMAT3 is a regulator of RNA-mediated gene silencing [27,29,36].

Next, we studied hypoxia induced changes in TP53-associated genes and their dependence on ERN1 activity. Interestingly, we have found that hypoxia affects the expression of many TP53-related genes and that hypoxia-induced changes in genes expression are similar to changes observed in glioma cells under basal conditions when ERN1 function is inhibited (Table 2, compare columns 2 and 3): increase in expression of RYBP, TP33BP2, and SESN1 and decrease in the expression of TOPORS and NME6 genes. Moreover, ERN1 participated in hypoxic regulation of the expression of TP53-associated genes. The inhibition of its function during hypoxia is associated with different fine-tuning of TP53-related genes (Table 2 compare columns 3 and 4, and Fig. 5,7 and 8).

Indeed, the expression of ZMAT3 and TP53 genes in glioma cells with ERN1 inhibition was resistant to hypoxia. Effect of hypoxia on the expression of TP53BP2 and TOPORS genes was significantly higher in glioma cells with inhibited ERN1 activity (Table 2, column 4). Therefore, we concluded that hypoxia leads to stabilization of TP53 and to enhance its activity, and that ERN1 participates in the fine-tuning of TP33BP2, TOPORS, and SESN1 genes expression during hypoxia. Since cells with dnERN1 has the magnitude of hypoxic effect and changes in expression of mentioned above genes is higher than in control cells. This correlates well with slower cell proliferation in cells harboring dnERN1. Endoplasmic reticulum stress and hypoxia are necessary components of malignant tumor growth and cell survival; a complex relationship exists between the presence of hypoxia and the regulation of cell death pathways [1,39-41], which warrants further investigation.

In conclusion, the results of this study clearly demonstrates that the expression of genes encoding the major TP53-related factors is dependent upon endoplasmic reticulum stress and hypoxic conditions, which correlates with suppression of cell proliferation in cells harboring dnERN1.

**Author contributions:** Minchenko DO, Danilovskyi SV and Minchenko OH contributed to conception and design, analysis and interpretation of data, wrote the paper; Kryvdiuk IV and Bakalets TV provided with a research technical support; Karbovskyi LL and Lypova NM revised the article critically for important intellectual content and final approval of the version to be published.

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| Gene   | Tested condition | dnERN1- cells versus control cells | Hypoxia versus regular growth in control cells | Hypoxia versus regular growth in dnERN1-cells |
|--------|------------------|-----------------------------------|-----------------------------------------------|------------------------------------------------|
| TP53   | Up               | Down                              | No change                                     |                                                |
| RYBP   | Up               | Down                              | Up-Up                                         |                                                |
| TP33BP1| Slightly down    | No change                         | Slightly up                                   |                                                |
| TP33BP2| Up               | No change                         | Up-Up                                         |                                                |
| SESN1  | Up               | No change                         | Up-Up                                         |                                                |
| TOPORS | Down             | Down                              | Down-Down                                     |                                                |
| NME6   | Down             | Down                              | Down-Down                                     |                                                |
| ZMAT3  | Down             | Down                              | No change                                     |                                                |
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