Published in final edited form as:

Arch Toxicol. 2017 February ; 91(2): 775–784. doi:10.1007/s00204-016-1746-3.

Up-regulation of Siah1 by ethanol triggers apoptosis in neural crest cells through p38 MAPK-mediated activation of p53 signaling pathway

Fuqiang Yuan^{1,2}, Xiaopan Chen^{1,2}, Jie Liu^{1,2}, Wenke Feng^{1,2,3}, Xiaoyang Wu⁴, and Shao-yu Chen^{1,2,*}

¹Department of Pharmacology and Toxicology, University of Louisville Health Sciences Center, Louisville, Kentucky 40292, USA

²University of Louisville Alcohol Research Center, Louisville, Kentucky 40292, USA

³Department of Medicine, University of Louisville, Kentucky 40292, USA

⁴Ben May Department for Cancer Research, University of Chicago, Chicago, IL 60637, USA

Abstract

Seven in absentia homolog 1 (SIAH1) is one of the E3 ubiquitin ligases and plays a key role in regulating target protein degradation. This study was designed to test the hypothesis that Siah1 mediates ethanol-induced apoptosis in NCCs through p38 MAPK-mediated activation of the p53 signaling pathway. We found that exposure of NCCs to ethanol resulted in the increases in the total protein levels of p53 and the phosphorylation of p53 at serine 15. Ethanol exposure also resulted in a significant increase in the phosphorylation of p38 MAPK. Knock-down of Siah1 dramatically reduced the ethanol-induced increase in the phosphorylation of p38 MAPK. Knock-down of Siah1 by siRNA or down-regulation of p38 MAPK by either siRNA or inhibitor significantly diminished ethanol-induced accumulations of p53 and the phosphorylation of p53. In addition, ethanol exposure resulted in a significant increase in the expression of p53 downstream targets and apoptosis in NCCs, which can be significantly diminished by down-regulation of Siah1 with siRNA. Knock-down of p38 MAPK by siRNA also dramatically reduced the ethanol-induced apoptosis. These results demonstrate that Siah1 plays a crucial role in ethanol-induced apoptosis in NCCs and that the up-regulation of Siah1 by ethanol can trigger apoptosis through p38 MAPK-mediated activation of the p53 signaling pathway.

Keywords

Siah1; ethanol; apoptosis; p53; p38 MAPK; Fetal Alcohol Spectrum Disorders

* Author to whom correspondence should be sent: Shao-yu Chen, Ph.D., Department of Pharmacology and Toxicology, University of Louisville Health Sciences Center, Louisville, KY 40292. Phone: (502) 852-8677. (502) 852-8927. shaoyu.chen@louisville.edu.

Conflict of interest statement: The authors declare no conflict of interest.

Introduction

Fetal Alcohol Spectrum Disorders (FASD) (Sokol et al. 2003) is a term which refers to a range of structural and functional birth defects caused by prenatal exposure to ethanol (Kodituwakku 2007). Studies have shown that excessive cell death in the selective cell population is one of the major mechanisms underlying ethanol-induced teratogenesis (Dunty et al. 2001; Naseer et al. 2014). Among these ethanol-sensitive cell populations is neural crest cells (NCCs), which is progenitor cells that can give rise to various cell types, such as multiple skeletal cells, neurons, glial cells, and mesenchymal cells (Delfino-Machin et al. 2007; Hall 2008). It has been demonstrated that ethanol exposure resulted in apoptosis in NCCs (Sulik et al. 1988; Sun et al. 2014) and that ethanol-induced apoptosis in NCCs contributes greatly to subsequent abnormalities that are phenotypic characteristics of FASD (Cartwright and Smith 1995; Kotch and Sulik 1992).

Multiple signaling pathways have been demonstrated to be involved in ethanol-induced apoptosis, including Bcl-2 family (Hong et al. 2002), Fas/Fas-L and p53 pathways (Jana et al. 2010), caspases (Han et al. 2005; Zhou et al. 2001), and MAPK signaling system (Morio et al. 2013). However, the molecular mechanisms underlying ethanol-induced apoptosis in embryonic cells have not been clearly elucidated. Recent studies in our laboratory have demonstrated that Seven in absentia homolog 1 (Siah1) is involved in ethanol-induced apoptosis (Sun et al. 2014).

Siah1, which is a mammalian homologue of *Drosophila* Sina (Carthew and Rubin 1990), belongs to the family of E3 ubiquitin ligases. Siah1 regulates the ubiquitination and degradation of a number of important substrates, including Kid (Germani et al. 2000), T-STAR (Venables et al. 2004), and HIPK2 (Kim et al. 2009b). Recent studies have also shown that β -catenin, a key component of Wnt/ β -catenin, can be degraded by Siah1-dependent ubiquitin proteasomal pathway (Leung et al. 2014; Yoshibayashi et al. 2007). Siah1 plays a prominent role in a variety of biological processes, including transcriptional control, mitosis, cell transformation, cell proliferation, cell migration and apoptosis (Germani et al. 2000; Kim et al. 2009b; Tanaka and Iino 2014; Venables et al. 2004; Xie et al. 2009). Our recent studies have also shown that Siah1 can be up-regulated by ethanol in NCCs and that knock-down of Siah1 by siRNA attenuated ethanol-induced apoptosis (Sun et al. 2014). However, the mechanisms by which Siah1 mediates ethanol-induced apoptosis in NCCs are not clearly understood.

It is well known that p38 MAPK is involved in the regulation of p53 in the apoptosis induced by UV (Chouinard et al. 2002) and other chemical reagents (Kwon et al. 2002; Lamy et al. 2011). In addition, the nuclear translocation of p53 can also be regulated by p38 MAPK pathway (Karunakaran et al. 2008). It has also been demonstrated that p38 MAPK directly phosphorylates p53 at the serine 15 and activates p53 (Chouinard et al. 2002; She et al. 2000). Studies have also shown that p38 MAPK pathway can be activated by ethanol treatment (Ku et al. 2007; Lee and Shukla 2007) and that a long-term exposure to a low concentration of ethanol up-regulated the phosphorylation of p38 MAPK in rats (Tian et al. 2014).

In the present study, an in vitro model of NCCs, JoMa1.3 cells was used to elucidate the mechanisms by which Siah1 mediates ethanol-induced apoptosis in NCCs. We found that the up-regulation of Siah1 by ethanol increased p38 MAPK phosphorylation. Ethanol-induced up-regulation of Siah1 also increased p53 stability and its phosphorylation in NCCs through the activation of p38 MAPK. We have also found that down-regulation of Siah1 significantly reduced ethanol-induced increases in the expression of p53 downstream targets and diminished ethanol-induced apoptosis in NCCs. These results demonstrate that the up-regulation of Siah1 by ethanol can trigger apoptosis through p38 MAPK-mediated activation of the p53 signaling pathway.

Material and methods

Cell culture and ethanol treatment

NCCs (JoMa1.3 cells) were cultured as described previously (Chen et al. 2013a; Maurer et al. 2007). Briefly, cells were grown on cell culture dishes coated with fibronectin and maintained in Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1) at 37°C in 5% CO₂ /95% air. For ethanol treatment, NCCs were treated with 100 mM ethanol for 24 hours. The stable ethanol levels were maintained by placing the cell culture plates in a plastic desiccator containing 100 mM ethanol in distilled water (Yan et al. 2010).

siRNA transfection

siRNA transfection was performed as described previously (Sun et al. 2014). Briefly, NCCs were transfected with ON-TARGETplus Siah1a siRNA (L-044891-01-0005), ON-TARGETplus Mapk14 siRNA (L-040125-00-0005) or ON-TARGETplus Non-targeting Pool (D-001810-10-20) (Thermo Scientific Dharmacon, Lafayette, CO, USA) at a final concentration of 60 nM using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA), following the manufacturer's instructions. The cells were harvested at 48 hours after transfection for experiments.

Western blotting

Western blotting was performed as described previously (Chen et al. 2013b; Sun et al. 2014). Proteins were probed with the following antibodies: SIAH1 rabbit pAb (Abcam, Cambridge, MA, USA), Phospho-p53 (Ser15) rabbit pAb (Cell Signaling, Beverly, MA, USA), p53 mouse mAb (Abcam, Cambridge, MA, USA), β -Actin mouse mAb (Santa Cruz, SantaCruz, CA, USA), Phospho-p38 MAPK (Thr180/Tyr182) rabbit mAb (Cell Signaling, Beverly, MA, USA), p38 MAPK rabbit pAb (Cell Signaling, Beverly, MA, USA), Phospho-MAPKAPK-2 (Thr222) antibody (Cell Signaling, Beverly, MA, USA), MAPKAPK-2 antibody (Cell Signaling, Beverly, MA, USA), PUMA rabbit pAb (Abcam, Cambridge, MA, USA), Bak rabbit pAb (Cell Signaling, Beverly, MA, USA) and Cleaved Caspase-3 (Asp175) rabbit pAb (Cell Signaling, Beverly, MA, USA). The membranes were developed on a Kodak X-OMAT 2000A imaging system (Kodak, Rochester, NY, USA). The intensity of the protein band was analyzed by ImageJ software (1.46b, National Institutes of Health, USA). All Western blot analyses were performed in triplicate.

Immunofluorescence analysis

Immunofluorescence analysis was performed as described previously (Sun et al. 2014). Cells grown on coverslips were fixed, permeabilized and blocked as described previously. Then the cells were incubated with 1:250 dilutions of the following primary antibodies: Siah1 goat pAb (Santa Cruz, SantaCruz, CA, USA), PUMA rabbit pAb (Abcam, Cambridge, MA, USA) and Bak rabbit pAb (Santa Cruz, SantaCruz, CA, USA). After washing with PBST three times, the cells were then incubated with 1:250 dilution of two secondary antibodies conjugated to different fluorochromes: Donkey Anti-Goat IgG H&L (Alexa Fluor® 647) and Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (Abcam, Cambridge, MA, USA). The cells were washed well with PBST and then counterstained with 0.2µg/µl DAPI for 1 min. and examined by a confocal laser microscope (Nikon A1 laser scanning confocal microscope).

Analysis of apoptosis

Apoptosis was determined by the analysis of caspase-3 cleavage and activity as well as the TUNEL assay. Caspase-3 cleavage was determined by Western blot as described previously (Chen et al. 2013a; Dong et al. 2008). Caspase-3 activity was determined by using Caspase-Glo® 3/7 Assay Systems (Promega, Madison, WI, USA). TUNEL assay was performed by using a TiterTACS In Situ Detection Kit (Trevigen, City, MD, USA), following the manufacturer's protocol.

Statistical analysis

Statistical analyses were performed as described previously (Chen et al. 2013b) using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). All data were expressed as means ± SEM of three separate experiments. One-way ANOVA was used to compare the difference between groups and multiple comparison posttests were conducted by using Bonferroni's test. Differences between groups were considered significant at $p < 0.05$.

Results

Up-regulation of Siah1 by ethanol resulted in increases in p53 stability and its phosphorylation in NCCs treated with ethanol

The p53 is a well-known protein involved in the induction of apoptosis (Kojima et al. 2005; Shao et al. 2000). To determine whether Siah1 mediates ethanol-induced apoptosis in NCCs through the activation of the p53 signaling pathway, we first determined whether ethanol exposure can increase p53 stability and its phosphorylation in NCCs. As shown in Fig. 1, exposure of NCCs to 100 mM ethanol for 24 hours significantly increased protein expression of Siah1, confirming our previous studies (Sun et al. 2014). Exposure to ethanol also resulted in a significant increase in p53 protein level in NCCs. In addition, ethanol treatment dramatically increased the phosphorylation of p53 at serine 15, indicating that ethanol exposure can activate p53 pathway in NCCs. To determine whether ethanol-mediated up-regulation of Siah1 is involved in p53 stability and phosphorylation in NCCs, cells transfected with control or Siah1-siRNA were exposed to ethanol. We found that knock-

down of Siah1 remarkably decreased ethanol-induced increases in p53 stability and its phosphorylation at serine 15 in NCCs, demonstrating that increases in p53 protein stability and phosphorylation in ethanol-exposed NCCs is mediated by the up-regulation of Siah1.

Up-regulation of Siah1 resulted in the activation of p38 MAPK in ethanol-exposed NCCs

The p38 MAPK pathway has been implicated in apoptosis induced by many stimuli, including ethanol (Ku et al. 2007; Lee and Shukla 2007). To determine whether ethanol exposure can result in the activation of p38 MAPK in NCCs, NCCs were treated with 100 mM ethanol for 24 hours. As shown in Fig. 2, Western blot analysis revealed a significant increase in the phosphorylation of p38 MAPK in NCCs exposed to ethanol, indicating that treatment with ethanol can significantly activate the p38 MAPK pathway in NCCs. We next determined whether ethanol-induced activation of p38 MAPK in NCCs was mediated by the up-regulation of Siah1. For this study, NCCs transfected with control or Siah1-siRNA were treated with ethanol. We found that knock-down of Siah1 by siRNA dramatically reduced the ethanol-induced increase in the phosphorylation of p38 MAPK, indicating that activation of p38 MAPK in ethanol-exposed NCCs is mediated, at least in part, by the up-regulation of Siah1 (Fig. 2).

Activation of p38 MAPK is involved in ethanol-induced Siah1-mediated activation of p53 pathway in NCCs

It is well known that p53 is one of the most important down-stream targets of p38 MAPK and that the phosphorylation of p53 and its activity are regulated by p38 MAPK pathway (Sanchez-Prieto et al. 2000). To determine whether ethanol-induced Siah1-mediated p53 pathway activation is mediated by ethanol-induced activation of p38 MAPK, NCCs were transfected with control or p38-siRNA, or treated with SB203580, a widely used p38 MAPK inhibitor (Elenitoba-Johnson et al. 2003; Sarkar et al. 2002). As shown in Fig. 3A, knock-down of p38 MAPK by siRNA significantly diminished ethanol-induced increases in the protein expression and the phosphorylation of p53, indicating that p38 activation is involved in ethanol-induced Siah1-mediated p53 activation in NCCs. This was supported by the fact that p38 MAPK inhibitor, SB203580, which can block the activation of MAPKAPK-2 by inhibiting p38 MAPK activity (Cuenda et al. 1995; Kumar et al. 1999), can also attenuate ethanol-induced p53 stability and phosphorylation (Fig. 3B).

Ethanol-induced up-regulation of Siah1 can induce transcriptional activity of p53 and increase the expression of pro-apoptotic protein PUMA and Bak

The p53 can act as a transcription factor for many pro-apoptotic genes, including p53-upregulated modulator of apoptosis (PUMA) (Nakano and Vousden 2001; Yu et al. 2003), which can activate Bak and induce apoptosis (Adlakha and Saini 2013; Dai et al. 2014; Kim et al. 2009a; Ren et al. 2010). To test whether Siah1 mediates ethanol-induced apoptosis in NCCs by activating the p53 pathway, we examined whether ethanol-induced upregulation of Siah1 can induce transcriptional activity of p53 by analyzing the expression of the p53 downstream pro-apoptotic protein, PUMA and Bak. We found that ethanol treatment resulted in a significant increase in the protein expression of PUMA and Bak, which can be attenuated by knock-down of Siah1 by siRNA (Fig. 4A). These results were further confirmed by the results from immunofluorescence analysis, which have shown that ethanol

exposure significantly increased the expression of Siah1, PUMA and Bak in NCCs and that knock-down of Siah1 by siRNA significantly reduced the ethanol-induced increase in the expression of PUMA and Bak (Fig. 4B).

Ethanol-induced up-regulation of Siah1 can trigger apoptosis in ethanol-exposed NCCs

To determine whether ethanol-induced up-regulation of Siah1 can trigger apoptosis in NCCs, ethanol-induced apoptosis in NCCs were determined by the analysis of caspase-3 cleavage and activity as well as the TUNEL assay. As shown in Fig. 5, ethanol treatment resulted in significant increases in caspase-3 cleavage and activity. Knockdown of Siah1 by siRNA greatly diminished ethanol-induced increases in the cleavage and activity of caspase-3 (Fig. 5A, B), indicating that ethanol-induced Siah1-mediated p53 activation contributes to ethanol-induced apoptosis in NCCs. This result was further confirmed by the analysis of apoptosis using TUNEL assay. As shown in Fig. 5C, ethanol-induced apoptosis was significantly diminished by Siah1-siRNA.

Activation of p38 MAPK is directly involved in the ethanol-induced Siah1-mediated apoptosis

To further confirm that activation of p38 MAPK is directly involved in the ethanol-induced Siah1-mediated apoptosis, ethanol-induced apoptosis were determined by the analysis of caspase-3 cleavage and activity as well as the TUNEL assay in NCCs transfected with control or p38-siRNA. As shown in Fig. 6, knock-down of p38 MAPK by siRNA greatly diminished ethanol-induced increases in the cleavage and activity of caspase-3 (Fig. 6A, B). TUNEL assay also confirmed that down-regulation of p38 MAPK significantly reduced the ethanol-induced apoptosis in NCCs (Fig. 6C), indicating that the ethanol-induced activation of p38 MAPK is directly involved in ethanol-induced Siah1-mediated apoptosis.

Discussion

Siah1 is a member of the family of E3 ubiquitin ligases that has been implicated in a variety of biological processes, including apoptosis. Our previous studies have shown that Siah1 can be up-regulated by ethanol in NCCs and that knock-down of Siah1 by siRNA can attenuate ethanol-induced apoptosis, indicating that Siah1 is involved in ethanol-induced apoptosis in NCCs (Sun et al. 2014). While it has been demonstrated by other investigators that Siah1 can promote apoptosis through different mechanisms, including JNK pathway (Wen et al. 2010b; Xu et al. 2006), beta-catenin signaling pathway (Yoshibayashi et al. 2007), and Bim (Wen et al. 2010a), the mechanisms by which Siah1 mediates ethanol-induced apoptosis in NCCs are not clearly understood. In this study, we found that the up-regulation of Siah1 by ethanol exposure resulted in the increases in p53 stability and its phosphorylation, and the activation of p38 MAPK in NCCs treated with ethanol. We also found that ethanol-induced Siah1-mediated activation of p53 pathway is mediated by the activation of p38 MAPK in NCCs. In addition, ethanol-induced upregulation of Siah1 can induce transcriptional activity of p53 and trigger apoptosis in ethanol-exposed NCCs.

It is well known that p53 protein is a powerful transcription factor that broadly regulates the expression of specific genes involved in apoptosis (Kho et al. 2004; Schuler and Green

2005). Recent studies have shown that knock-down of Siah1 inhibited p53 phosphorylation and reduced Bax protein levels in cells exposed to high glucose (Yego and Mohr 2010). In this study, we found that knock-down of Siah1 remarkably decreased ethanol-induced increases in p53 stability and its phosphorylation at serine 15 in NCCs, demonstrating that increases in p53 protein stability and phosphorylation in ethanol-exposed NCCs is mediated by the up-regulation of Siah1. It is noteworthy that previous studies have shown that Siah1 is a downstream effector of p53 and that the expression of Siah1 can be induced by over-expression of p53 (Amson et al. 1996; Liu et al. 2001; Relaix et al. 2000), suggesting that Siah1 and p53 may regulate via a positive feedback mechanism to promote apoptosis in ethanol-exposed NCCs.

The p38 MAPK pathway has been implicated in apoptosis induced by many stimuli through the regulation of p53 and its downstream effectors, eventually leading to apoptosis (Gao et al. 2014; Zheng et al. 2005). Studies have shown that p38 MAPK pathway can be activated by ethanol treatment (Ku et al. 2007; Lee and Shukla 2007). However, the mechanism by which ethanol activates p38 MAPK is not clearly understood. In this study, we have demonstrated that ethanol exposure induced the activation of p38 MAPK in NCCs and that knock-down of Siah1 by siRNA dramatically reduced the ethanol-induced increase in the phosphorylation of p38 MAPK, indicating that activation of p38 MAPK in ethanol-exposed NCCs is mediated, at least in part, by the up-regulation of Siah1.

It is clear that posttranslational modification of p53 plays an important role in its stabilization and activation. Studies have shown that the phosphorylation of p53 at its serine 15 and 37 interferes with the interaction between p53 and MDM2, leading to the accumulation of p53 (Shieh et al. 1997). p53 is considered to be one of the most important down-stream targets of p38 MAPK. It has been found that activation of p38 MAPK is sufficient to promote p53 function and that inhibition of p38 MAPK prevented the induction of the transcriptional activity of p53 (Sanchez-Prieto et al. 2000). In the present study, we found that knock-down of p38 MAPK by either siRNA or its inhibitor significantly decreased ethanol-induced accumulation and phosphorylation of p53, suggesting that p38 activation is essential for ethanol-induced Siah1-mediated p53 activation in NCCs.

p53 is involved in the induction of various types of apoptosis by acting as a transcription factor for many pro-apoptotic genes, including PUMA (Fridman and Lowe 2003). PUMA is one of the most potent killers among the Bcl-2 family members and is considered to account for virtually all of the pro-apoptotic activity of p53 (Jeffers et al. 2003; Yu and Zhang 2008). Studies have shown that chronic ethanol feeding in rats resulted in p53 activation and increases in oxidative stress and PUMA expression, leading to apoptosis in the liver (Derdak et al. 2011). In this study, we found that ethanol exposure resulted in significant increases in the protein expression and phosphorylation of p53, accumulation of PUMA, the activation of caspase-3 and apoptosis in NCCs, which can be significantly diminished by down-regulation of Siah1, indicating that up-regulation of Siah1 in ethanol-exposed NCCs can induce apoptosis by activation of p53 signaling pathways. Knock-down of p38 MAPK by siRNA also dramatically reduced the ethanol-induced apoptosis. These results demonstrate that the up-regulation of Siah1 by ethanol can trigger apoptosis through p38 MAPK-mediated activation of the p53 signaling pathway.

In conclusion, the present study confirmed that Siah1 is involved in ethanol-induced apoptosis in NCCs. We have also demonstrated that ethanol treatment resulted in a significant increase in the phosphorylation of p38 by upregulation of Siah1 and that p38 activation is essential for ethanol-induced Siah1-mediated p53 activation in NCCs. These results demonstrate that Siah1 plays a crucial role in ethanol-induced apoptosis in NCCs and that the upregulation of Siah1 by ethanol can trigger apoptosis through p38 MAPK-mediated activation of the p53 signaling pathway. These findings elucidate the mechanisms by which Siah1 mediates ethanol-induced apoptosis in NCCs and may help to develop novel approaches to preventing FASD based on modulation of the Siah1-dependent apoptotic cascade.

Acknowledgments

This work was supported by the National Institute of Health grants AA020265, AA021434 (S.-YC), AA020848, and AA022416 (W. F) from the National Institute on Alcohol Abuse and Alcoholism and AR063630 (X. W) from the National Institute of Arthritis and Musculoskeletal and Skin Diseases.

Abbreviations

Siah1	Seven in absentia homolog 1
NCCs	neural crest cells
FASD	Fetal Alcohol Spectrum Disorders
MAPK	mitogen-activated protein kinases
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
PUMA	p53-upregulated modulator of apoptosis

References

- Adlakha YK, Saini N. miR-128 exerts pro-apoptotic effect in a p53 transcription-dependent and -independent manner via PUMA-Bak axis. *Cell death & disease*. 2013; 4:e542.doi: 10.1038/cddis.2013.46 [PubMed: 23492773]
- Amson RB, Nemani M, Roperch JP, et al. Isolation of 10 differentially expressed cDNAs in p53-induced apoptosis: activation of the vertebrate homologue of the drosophila seven in absentia gene. *Proceedings of the National Academy of Sciences of the United States of America*. 1996; 93(9): 3953–7. [PubMed: 8632996]
- Carthew RW, Rubin GM. seven in absentia, a gene required for specification of R7 cell fate in the *Drosophila* eye. *Cell*. 1990; 63(3):561–77. [PubMed: 2146028]
- Cartwright MM, Smith SM. Increased cell death and reduced neural crest cell numbers in ethanol-exposed embryos: partial basis for the fetal alcohol syndrome phenotype. *Alcoholism, clinical and experimental research*. 1995; 19(2):378–86.
- Chen X, Liu J, Chen SY. Over-expression of Nrf2 diminishes ethanol-induced oxidative stress and apoptosis in neural crest cells by inducing an antioxidant response. *Reproductive toxicology*. 2013a; 42:102–9. DOI: 10.1016/j.reprotox.2013.08.003 [PubMed: 23994065]
- Chen X, Liu J, Chen SY. Sulforaphane protects against ethanol-induced oxidative stress and apoptosis in neural crest cells by the induction of Nrf2-mediated antioxidant response. *British journal of pharmacology*. 2013b; 169(2):437–48. DOI: 10.1111/bph.12133 [PubMed: 23425096]
- Chouinard N, Valerie K, Rouabhia M, Huot J. UVB-mediated activation of p38 mitogen-activated protein kinase enhances resistance of normal human keratinocytes to apoptosis by stabilizing

- cytoplasmic p53. *The Biochemical journal*. 2002; 365(Pt 1):133–45. DOI: 10.1042/BJ20020072 [PubMed: 12071847]
- Cuenda A, Rouse J, Doza YN, et al. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS letters*. 1995; 364(2):229–33. [PubMed: 7750577]
- Dai H, Pang YP, Ramirez-Alvarado M, Kaufmann SH. Evaluation of the BH3-only protein Puma as a direct Bak activator. *The Journal of biological chemistry*. 2014; 289(1):89–99. DOI: 10.1074/jbc.M113.505701 [PubMed: 24265320]
- Delfino-Machin M, Chipperfield TR, Rodrigues FS, Kelsh RN. The proliferating field of neural crest stem cells. *Developmental dynamics : an official publication of the American Association of Anatomists*. 2007; 236(12):3242–54. DOI: 10.1002/dvdy.21314 [PubMed: 17823935]
- Derdak Z, Lang CH, Villegas KA, et al. Activation of p53 enhances apoptosis and insulin resistance in a rat model of alcoholic liver disease. *Journal of hepatology*. 2011; 54(1):164–72. DOI: 10.1016/j.jhep.2010.08.007 [PubMed: 20961644]
- Dong J, Sulik KK, Chen SY. Nrf2-mediated transcriptional induction of antioxidant response in mouse embryos exposed to ethanol in vivo: implications for the prevention of fetal alcohol spectrum disorders. *Antioxidants & redox signaling*. 2008; 10(12):2023–33. DOI: 10.1089/ars.2007.2019 [PubMed: 18759561]
- Dunty WC Jr, Chen SY, Zucker RM, Dehart DB, Sulik KK. Selective vulnerability of embryonic cell populations to ethanol-induced apoptosis: implications for alcohol-related birth defects and neurodevelopmental disorder. *Alcoholism, clinical and experimental research*. 2001; 25(10):1523–35.
- Elenitoba-Johnson KS, Jenson SD, Abbott RT, et al. Involvement of multiple signaling pathways in follicular lymphoma transformation: p38-mitogen-activated protein kinase as a target for therapy. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100(12):7259–64. DOI: 10.1073/pnas.1137463100 [PubMed: 12756297]
- Fridman JS, Lowe SW. Control of apoptosis by p53. *Oncogene*. 2003; 22(56):9030–40. DOI: 10.1038/sj.onc.1207116 [PubMed: 14663481]
- Gao J, Gao J, Qian L, et al. Activation of p38-MAPK by CXCL4/CXCR3 axis contributes to p53-dependent intestinal apoptosis initiated by 5-fluorouracil. *Cancer biology & therapy*. 2014; 15(8):982–91. DOI: 10.4161/cbt.29114 [PubMed: 24800927]
- Germani A, Bruzzoni-Giovanelli H, Fellous A, Gisselbrecht S, Varin-Blank N, Calvo F. SIAH-1 interacts with alpha-tubulin and degrades the kinesin Kid by the proteasome pathway during mitosis. *Oncogene*. 2000; 19(52):5997–6006. DOI: 10.1038/sj.onc.1204002 [PubMed: 11146551]
- Hall BK. The neural crest and neural crest cells: discovery and significance for theories of embryonic organization. *Journal of biosciences*. 2008; 33(5):781–93. [PubMed: 19179766]
- Han JY, Joo Y, Kim YS, et al. Ethanol induces cell death by activating caspase-3 in the rat cerebral cortex. *Molecules and cells*. 2005; 20(2):189–95. [PubMed: 16267392]
- Hong F, Kim WH, Tian Z, et al. Elevated interleukin-6 during ethanol consumption acts as a potential endogenous protective cytokine against ethanol-induced apoptosis in the liver: involvement of induction of Bcl-2 and Bcl-x(L) proteins. *Oncogene*. 2002; 21(1):32–43. DOI: 10.1038/sj.onc.1205016 [PubMed: 11791174]
- Jana K, Jana N, De DK, Guha SK. Ethanol induces mouse spermatogenic cell apoptosis in vivo through overexpression of Fas/Fas-L, p53, and caspase-3 along with cytochrome c translocation and glutathione depletion. *Molecular reproduction and development*. 2010; 77(9):820–33. DOI: 10.1002/mrd.21227 [PubMed: 20803734]
- Jeffers JR, Parganas E, Lee Y, et al. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer cell*. 2003; 4(4):321–8. [PubMed: 14585359]
- Karunakaran S, Saeed U, Mishra M, et al. Selective activation of p38 mitogen-activated protein kinase in dopaminergic neurons of substantia nigra leads to nuclear translocation of p53 in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2008; 28(47):12500–9. DOI: 10.1523/JNEUROSCI.4511-08.2008 [PubMed: 19020042]

- Kho PS, Wang Z, Zhuang L, et al. p53-regulated transcriptional program associated with genotoxic stress-induced apoptosis. *The Journal of biological chemistry*. 2004; 279(20):21183–92. DOI: 10.1074/jbc.M311912200 [PubMed: 15016801]
- Kim H, Tu HC, Ren D, et al. Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. *Molecular cell*. 2009a; 36(3):487–99. DOI: 10.1016/j.molcel.2009.09.030 [PubMed: 19917256]
- Kim SY, Choi DW, Kim EA, Choi CY. Stabilization of HIPK2 by escape from proteasomal degradation mediated by the E3 ubiquitin ligase Siah1. *Cancer letters*. 2009b; 279(2):177–84. DOI: 10.1016/j.canlet.2009.01.036 [PubMed: 19250734]
- Kodituwakku PW. Defining the behavioral phenotype in children with fetal alcohol spectrum disorders: a review. *Neuroscience and biobehavioral reviews*. 2007; 31(2):192–201. DOI: 10.1016/j.neubiorev.2006.06.020 [PubMed: 16930704]
- Kojima K, Konopleva M, Samudio IJ, et al. MDM2 antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy. *Blood*. 2005; 106(9):3150–9. DOI: 10.1182/blood-2005-02-0553 [PubMed: 16014563]
- Kotch LE, Sulik KK. Experimental fetal alcohol syndrome: proposed pathogenic basis for a variety of associated facial and brain anomalies. *American journal of medical genetics*. 1992; 44(2):168–76. DOI: 10.1002/ajmg.1320440210 [PubMed: 1456286]
- Ku BM, Lee YK, Jeong JY, et al. Ethanol-induced oxidative stress is mediated by p38 MAPK pathway in mouse hippocampal cells. *Neuroscience letters*. 2007; 419(1):64–7. DOI: 10.1016/j.neulet.2007.03.049 [PubMed: 17420100]
- Kumar S, Jiang MS, Adams JL, Lee JC. Pyridinylimidazole compound SB 203580 inhibits the activity but not the activation of p38 mitogen-activated protein kinase. *Biochemical and biophysical research communications*. 1999; 263(3):825–31. DOI: 10.1006/bbrc.1999.1454 [PubMed: 10512765]
- Kwon YW, Ueda S, Ueno M, Yodoi J, Masutani H. Mechanism of p53-dependent apoptosis induced by 3-methylcholanthrene: involvement of p53 phosphorylation and p38 MAPK. *The Journal of biological chemistry*. 2002; 277(3):1837–44. DOI: 10.1074/jbc.M105033200 [PubMed: 11706017]
- Lamy V, Bousserouel S, Gosse F, Minker C, Lobstein A, Raul F. Lupulone triggers p38 MAPK-controlled activation of p53 and of the TRAIL receptor apoptotic pathway in human colon cancer-derived metastatic cells. *Oncology reports*. 2011; 26(1):109–14. DOI: 10.3892/or.2011.1273 [PubMed: 21519792]
- Lee YJ, Shukla SD. Histone H3 phosphorylation at serine 10 and serine 28 is mediated by p38 MAPK in rat hepatocytes exposed to ethanol and acetaldehyde. *European journal of pharmacology*. 2007; 573(1–3):29–38. DOI: 10.1016/j.ejphar.2007.06.049 [PubMed: 17643407]
- Leung CO, Deng W, Ye TM, et al. miR-135a leads to cervical cancer cell transformation through regulation of beta-catenin via a SIAH1-dependent ubiquitin proteasomal pathway. *Carcinogenesis*. 2014; 35(9):1931–40. DOI: 10.1093/carcin/bgu032 [PubMed: 24503442]
- Liu J, Stevens J, Rote CA, et al. Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. *Molecular cell*. 2001; 7(5):927–36. [PubMed: 11389840]
- Maurer J, Fuchs S, Jager R, Kurz B, Sommer L, Schorle H. Establishment and controlled differentiation of neural crest stem cell lines using conditional transgenesis. *Differentiation; research in biological diversity*. 2007; 75(7):580–91. DOI: 10.1111/j.1432-0436.2007.00164.x [PubMed: 17381545]
- Morio Y, Tsuji M, Inagaki M, et al. Ethanol-induced apoptosis in human liver adenocarcinoma cells (SKHep1): Fas- and mitochondria-mediated pathways and interaction with MAPK signaling system. *Toxicology in vitro : an international journal published in association with BIBRA*. 2013; 27(6):1820–9. DOI: 10.1016/j.tiv.2013.05.009 [PubMed: 23726865]
- Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. *Molecular cell*. 2001; 7(3):683–94. [PubMed: 11463392]
- Naseer MI, Ullah I, Rasool M, et al. Downregulation of dopamine D(1) receptors and increased neuronal apoptosis upon ethanol and PTZ exposure in prenatal rat cortical and hippocampal neurons. *Neurological sciences : official journal of the Italian Neurological Society and of the*

- Italian Society of Clinical Neurophysiology. 2014; 35(11):1681–8. DOI: 10.1007/s10072-014-1812-7
- Relaix F, Wei X, Li W, et al. Pw1/Peg3 is a potential cell death mediator and cooperates with Siah1a in p53-mediated apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97(5):2105–10. DOI: 10.1073/pnas.040378897 [PubMed: 10681424]
- Ren D, Tu HC, Kim H, et al. BID, BIM, and PUMA are essential for activation of the BAX- and BAK-dependent cell death program. *Science*. 2010; 330(6009):1390–3. DOI: 10.1126/science.1190217 [PubMed: 21127253]
- Sanchez-Prieto R, Rojas JM, Taya Y, Gutkind JS. A role for the p38 mitogen-activated protein kinase pathway in the transcriptional activation of p53 on genotoxic stress by chemotherapeutic agents. *Cancer research*. 2000; 60(9):2464–72. [PubMed: 10811125]
- Sarkar D, Su ZZ, Lebedeva IV, et al. mda-7 (IL-24) Mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99(15):10054–9. DOI: 10.1073/pnas.152327199 [PubMed: 12114539]
- Schuler M, Green DR. Transcription, apoptosis and p53: catch-22. *Trends in genetics : TIG*. 2005; 21(3):182–7. DOI: 10.1016/j.tig.2005.01.001 [PubMed: 15734577]
- Shao J, Fujiwara T, Kadowaki Y, et al. Overexpression of the wild-type p53 gene inhibits NF-kappaB activity and synergizes with aspirin to induce apoptosis in human colon cancer cells. *Oncogene*. 2000; 19(6):726–36. DOI: 10.1038/sj.onc.1203383 [PubMed: 10698490]
- She QB, Chen N, Dong Z. ERKs and p38 kinase phosphorylate p53 protein at serine 15 in response to UV radiation. *The Journal of biological chemistry*. 2000; 275(27):20444–9. DOI: 10.1074/jbc.M001020200 [PubMed: 10781582]
- Shieh SY, Ikeda M, Taya Y, Prives C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell*. 1997; 91(3):325–34. [PubMed: 9363941]
- Sokol RJ, Delaney-Black V, Nordstrom B. Fetal alcohol spectrum disorder. *Jama*. 2003; 290(22):2996–9. DOI: 10.1001/jama.290.22.2996 [PubMed: 14665662]
- Sulik KK, Cook CS, Webster WS. Teratogens and craniofacial malformations: relationships to cell death. *Development*. 1988; 103(Suppl):213–31. [PubMed: 3074910]
- Sun H, Chen X, Yuan F, Liu J, Zhao Y, Chen SY. Involvement of seven in absentia homolog-1 in ethanol-induced apoptosis in neural crest cells. *Neurotoxicology and teratology*. 2014; 46C:26–31. DOI: 10.1016/j.ntt.2014.08.006
- Tanaka T, Iino M. Sec6 regulated cytoplasmic translocation and degradation of p27 via interactions with Jab1 and Siah1. *Cellular signalling*. 2014; 26(10):2071–85. DOI: 10.1016/j.cellsig.2014.06.003 [PubMed: 24949832]
- Tian C, Jin X, Ye X, et al. Long term intake of 0.1% ethanol decreases serum adiponectin by suppressing PPARgamma expression via p38 MAPK pathway. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 2014; 65:329–34. DOI: 10.1016/j.fct.2014.01.007 [PubMed: 24412557]
- Venables JP, Dalglish C, Paronetto MP, et al. SIAH1 targets the alternative splicing factor T-STAR for degradation by the proteasome. *Human molecular genetics*. 2004; 13(14):1525–34. DOI: 10.1093/hmg/ddh165 [PubMed: 15163637]
- Wen YY, Yang ZQ, Song M, et al. The expression of SIAH1 is downregulated and associated with Bim and apoptosis in human breast cancer tissues and cells. *Molecular carcinogenesis*. 2010a; 49(5):440–9. DOI: 10.1002/mc.20615 [PubMed: 20082325]
- Wen YY, Yang ZQ, Song M, Li BL, Zhu JJ, Wang EH. SIAH1 induced apoptosis by activation of the JNK pathway and inhibited invasion by inactivation of the ERK pathway in breast cancer cells. *Cancer science*. 2010b; 101(1):73–9. DOI: 10.1111/j.1349-7006.2009.01339.x [PubMed: 19775288]
- Xie W, Jin L, Mei Y, Wu M. E2F1 represses beta-catenin/TCF activity by direct up-regulation of Siah1. *Journal of cellular and molecular medicine*. 2009; 13(8B):1719–27. [PubMed: 20187294]
- Xu Z, Sproul A, Wang W, Kukekov N, Greene LA. Siah1 interacts with the scaffold protein POSH to promote JNK activation and apoptosis. *The Journal of biological chemistry*. 2006; 281(1):303–12. DOI: 10.1074/jbc.M509060200 [PubMed: 16230351]

- Yan D, Dong J, Sulik KK, Chen SY. Induction of the Nrf2-driven antioxidant response by tert-butylhydroquinone prevents ethanol-induced apoptosis in cranial neural crest cells. *Biochemical pharmacology*. 2010; 80(1):144–9. DOI: 10.1016/j.bcp.2010.03.004 [PubMed: 20223225]
- Yego EC, Mohr S. Siah-1 Protein is necessary for high glucose-induced glyceraldehyde-3-phosphate dehydrogenase nuclear accumulation and cell death in Muller cells. *The Journal of biological chemistry*. 2010; 285(5):3181–90. DOI: 10.1074/jbc.M109.083907 [PubMed: 19940145]
- Yoshibayashi H, Okabe H, Satoh S, et al. SIAH1 causes growth arrest and apoptosis in hepatoma cells through beta-catenin degradation-dependent and -independent mechanisms. *Oncology reports*. 2007; 17(3):549–56. [PubMed: 17273732]
- Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100(4):1931–6. DOI: 10.1073/pnas.2627984100 [PubMed: 12574499]
- Yu J, Zhang L. PUMA, a potent killer with or without p53. *Oncogene*. 2008; 27(Suppl 1):S71–83. DOI: 10.1038/onc.2009.45 [PubMed: 19641508]
- Zheng SY, Fu XB, Xu JG, Zhao JY, Sun TZ, Chen W. Inhibition of p38 mitogen-activated protein kinase may decrease intestinal epithelial cell apoptosis and improve intestinal epithelial barrier function after ischemia- reperfusion injury. *World journal of gastroenterology : WJG*. 2005; 11(5): 656–60. [PubMed: 15655816]
- Zhou Z, Sun X, Kang YJ. Ethanol-induced apoptosis in mouse liver: Fas- and cytochrome c-mediated caspase-3 activation pathway. *The American journal of pathology*. 2001; 159(1):329–38. DOI: 10.1016/S0002-9440(10)61699-9 [PubMed: 11438480]

Fig. 1. Up-regulation of Siah1 by ethanol significantly increased p53 stability and its phosphorylation in NCCs

NCCs transfected with control siRNA or Siah1-siRNA were treated with 100 mM ethanol for 24 hours. The expression of Siah1, total p53 and phosphorylated p53 at serine 15 was determined by western blot. Data are expressed as fold change over control and represent the mean $\pm$ SEM of three separate experiments. * $p < 0.05$ vs. control; # $p < 0.05$ vs. ethanol

Fig. 2. Up-regulation of Siah1 resulted in the activation of p38 MAPK in ethanol-exposed NCCs NCCs transfected with control siRNA or Siah1-siRNA was treated with 100 mM ethanol for 24 hours. The expression of Siah1, total p38 and phosphorylated p38 was determined by western blot. Data are expressed as fold change over control and represent the mean $\pm$ SEM of three separate experiments. * $p < 0.05$ vs. control; # $p < 0.05$ vs. ethanol

Fig. 3. Activation of p38 MAPK is involved in ethanol-induced Siah1-mediated activation of p53 pathway in NCCs

A. NCCs transfected with control siRNA or p38-siRNA were treated with 100 mM ethanol for 24 hours. The expression of total p38, total p53 and phosphorylated p53 at serine 15 was determined by western blot. B. NCCs were pretreated with or without 10 μ M p38 inhibitor, SB203580, for 1 hour followed by the exposure to 100 mM ethanol for 24 hours. The expression of phospho MAPKAPK-2, total MAPKAPK-2, total p53 and phosphorylated p53 was determined by western blot. Data are expressed as fold change over control and

represent the mean $\pm$ SEM of three separate experiments. * $p < 0.05$ vs. control; # $p < 0.05$ vs. ethanol

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5. Ethanol-induced up-regulation of Siah1 can trigger apoptosis in NCCs

NCCs transfected with control siRNA or Siah1-siRNA were treated with 100 mM ethanol for 24 hours. Ethanol-induced apoptosis was determined by analysis of the cleavage (A) and activity (B) of caspase-3 as well as TUNEL analysis (C) as described in the Methods section. Data are expressed as fold change over control and represent the mean $\pm$ SEM of three separate experiments. * $p < 0.05$ vs. control; # $p < 0.05$ vs. ethanol