Biochemical and Biophysical Research Communications 438 (2013) 607–612

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Arsenic-induced cutaneous hyperplastic lesions are associated with the dysregulation of Yap, a Hippo signaling-related protein

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ARTICLE INFO

Article history:
Received 30 July 2013
Available online 11 August 2013

Keywords:
Arsenic
Hippo signaling pathway
Yap
Carcinogenesis

ABSTRACT

Arsenic exposure in humans causes a number of toxic manifestations in the skin including cutaneous neoplasm. However, the mechanism of these alterations remains elusive. Here, we provide novel observations that arsenic induced Hippo signaling pathway in the murine skin. This pathway plays crucial roles in determining organ size during the embryonic development and if aberrantly activated in adults, contributes to the pathogenesis of epithelial neoplasm. Arsenic treatment enhanced phosphorylation-dependent activation of LATS1 kinase and other Hippo signaling regulatory proteins Sav1 and MOB1. Phospho-LATS kinase is known to catalyze the inactivation of a transcriptional co-activator, Yap. However, in arsenic-treated epidermis, we did not observed its inactivation. Thus, as expected, unphosphorylated-Yap was translocated to the nucleus in arsenic-treated epidermis. Yap by binding to the transcription factors TEADs induces transcription of its target genes. Consistently, an up-regulation of Yap-dependent target genes Cyr61, Gli2, Ankrd1 and Ctgf was observed in the skin of arsenic-treated mice. Phosphorylated Yap is important in regulating tight and adherens junctions through its binding to αCatenin. We found disruption of these junctions in the arsenic-treated mouse skin despite an increase in αCatenin. These data provide evidence that arsenic-induced canonical Hippo signaling pathway and Yap-mediated disruption of tight and adherens junctions are independently regulated. These effects together may contribute to the carcinogenic effects of arsenic in the skin.

1. Introduction

Arsenic exposure through drinking water is a major global public health problem [1]. Approximately 150 million people are exposed to the toxic levels of arsenic worldwide including Bangladesh, Taiwan, Mexico, Mongolia, Argentina, India, Chile, etc. In some parts of the United States of America, high concentrations of arsenic are found in underground water [2]. Exposure to arsenic is associated with the enhanced risk of cancers in various organs including bladder, kidney, lung, liver and skin [3]. In humans, chronic arsenic exposure induces a dry skin phenotype, melanosis, hyperplasia and hyperkeratosis. Some of the precancerous lesions may ultimately progress to basal cell carcinoma (BCC) or squamous cell carcinoma (SCC) [4]. However, these changes in murine models have so far been not described and the mechanism by which arsenic induces these pathological alterations remains elusive.

Hippo signaling pathway is an evolutionarily conserved cascade that controls organ size by regulating cell proliferation, differentiation, apoptosis, and stem cell self renewal [5]. The core Hippo signaling pathway consists of a kinase cascade in which mammalian STE20-like kinase 1/2 (Mst1/2) and Salvador homolog 1 (Sav1) form a complex which phosphorylates and activates a downstream kinase Large tumor suppressor kinase 1/2 (LATS1/2). The regulatory protein MOB kinase activator 1A (MOB1) forms complex with the active LATS1/2 to phosphorylate its downstream transcription co-activators Yes-associated protein (Yap) and transcriptional co-activator with PDZ-binding motif (TAZ). This leads to the inhibition of their activities via their cytoplasmic retention or proteasomal degradation [5]. However, unphosphorylated-Yap and TAZ translocate into the nucleus and interact with transcription factors Tea-domain (TEAD) to induce the expression of their target genes, which were shown to be involved in cell proliferation and apoptosis inhibition [6]. The upstream regulators of core kinase cascade Mst1/2-LATS1/2-Yap/TAZ include proteins such as Merlin, KIBRA, RASSFs, and Ajuba [5]. However, increasing evidence indicates that αCatenin or ZO-2 may independently regulate Yap/TAZ at the adherens junctions and/or tight junctions [7,8]. In addition, the

Abbreviations: Yap, Yes-associated protein; Mst1/2, mammalian STE20-like kinase 1/2; Sav1, Salvador homolog 1; LATS1/2, large tumor suppressor kinase 1/2; MOB1, MOB kinase activator 1A; TAZ, transcriptional co-activator with PDZ-binding motif; TEAD, Tea-domain; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; AMOTL1, Angiomotin-like 1.

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http://dx.doi.org/10.1016/j.bbrc.2013.08.008

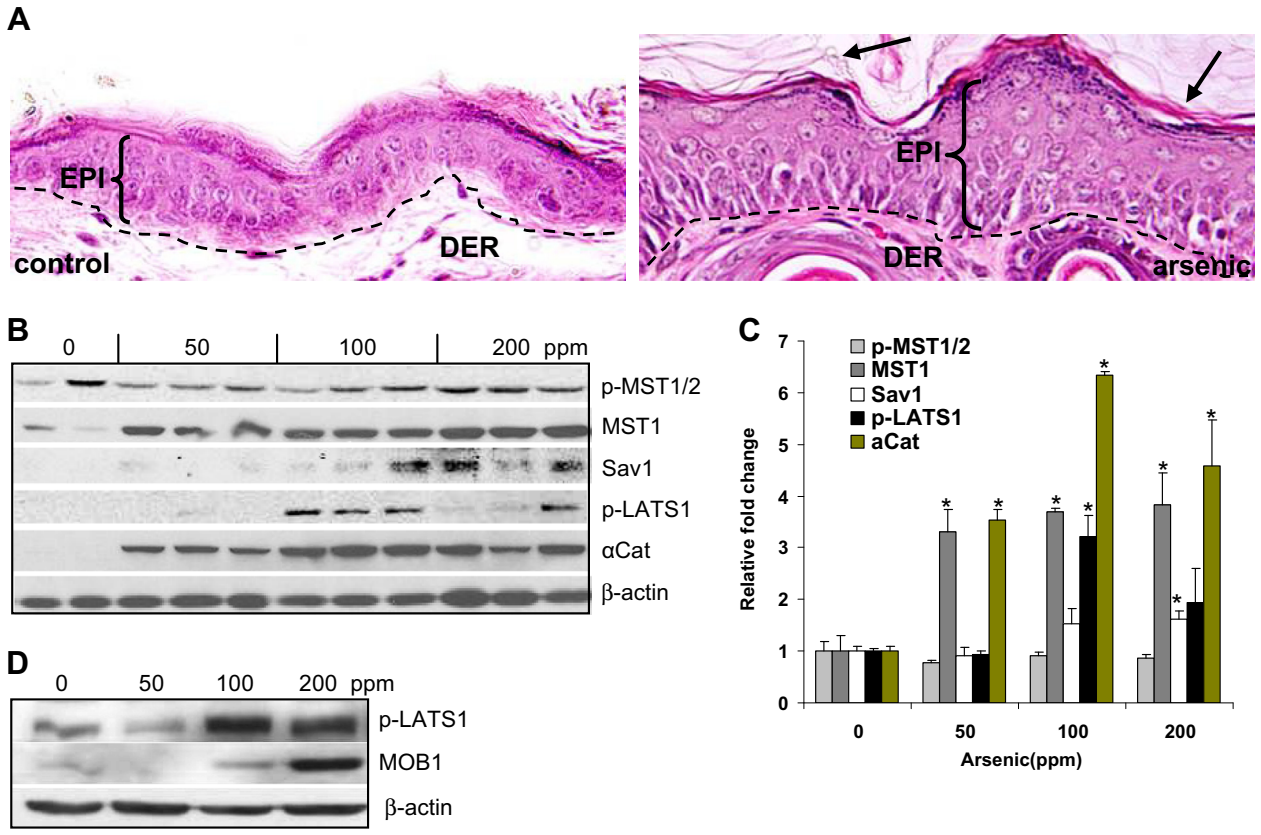

Fig. 1. Arsenic up-regulates Hippo signaling in murine skin. (A) Histological pictures showing arsenic exposure leads to hyperkeratosis, hyperplasia and epidermis disorganization in the skin of SKH-1 mice. Skin samples shown here were from control and 200 ppm sodium arsenite treatment group. Black arrows indicate the stratum corneum. Note that the thickness of stratum corneum was significantly augmented in the focal areas of arsenic-treated skin. The dashed lines delineate the boarder of epidermis and dermis. The braces indicate the thickness of epidermis. Each picture is representative of three independent skin samples. EPI stands for epidermis and DER stands for dermis. (B) & (C) Western blot and statistical analysis showing the expression of p-MST1/2, MST1, Sav1, p-LATS1 and αCatenin in the skin of SKH-1 mice. *indicates $p < 0.05$ when compared to control. Bars represent mean ± SEM ($n = 3$). (D) Western blot analysis showing arsenic up-regulates the expression of p-LATS1 and MOB1 in the skin of SKH-1 mice. For this, three skin samples from one group were pooled together, thus the individual band density represents the average value of protein expression level for each group.

activities of Mst1/2, LATS1/2 and Yap/TAZ may also be regulated by phosphatases, ubiquitination and by the cytoskeleton proteins [8–11]. Dysregulation of the Hippo pathway can lead to cancer development in various organs including skin [5].

In this study, we show for the first time that arsenic activates Hippo signaling pathway in the skin. Treatment of SKH-1 hairless mice with arsenic up-regulates αCatenin without inducing Yap phosphorylation. Instead, arsenic independently activates Yap, leading to its nuclear translocation and transcriptional activation. These data provide a novel mechanism by which some of the cutaneous manifestations of arsenic toxicity and carcinogenicity could be mediated.

2. Materials and methods

2.1. Reagents

Primary antibodies: αCatenin (sc-7894, Santa Cruz, Dallas, TX), MOB1 (3863, Cell Signaling, Danvers, MA), p-MST1/2 (3681, Cell signaling), MST1 (3682, Cell Signaling), p-LATS1 (9157s, Cell Signaling), Yap (4912, Cell Signaling), p-Yap (4911s, Cell Signaling), Sav1 (3507, Cell Signaling), TAZ (4883s, Cell signaling), β-actin (A-5316, Sigma, St. Louis, MO) were purchased.

2.2. Animals

To study the effects of arsenic on Hippo signaling, we utilized the skin samples obtained from the study published earlier [12]. Briefly, 25 age-matched SKH-1 hairless mice (5 mice/group) were fed *ad libitum* respectively drinking water containing arsenic at 0 ppm, 50 ppm, 100 ppm and 200 ppm concentrations for a period of 1 month. Then all of these animals were killed, their skin excised and processed for histology/immunohistochemistry/immunofluorescence studies or Western blot/PCR analysis. All experimental procedures involving animals were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

2.3. Western blot

Western blots were performed as previously described [12]. Briefly, skin tissues were homogenized in an ice-cold lysis buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 0.25% NaF, 10 mM β-glycerolphosphate, 2 mM EDTA, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 10 mM DTT and protease inhibitor). Clear lysate was prepared by centrifugation at 10,000*g* for 10 min. Proteins were denatured in 4× loading buffer, subjected to SDS–PAGE and electrophoretically transferred to PVDF membranes. The nonspecific sites were blocked with 5% (W/V) nonfat-dry milk in TBST for 1 h at RT. Blots were probed with primary antibody (4 °C overnight) followed by incubation with HRP-conjugated secondary antibody (1 h at RT). Then the blots were developed with Western blotting luminol reagent (sc-2048, Santa cruz). The integrated density of bands was measured with Image J software (http://rsb.info.nih.gov/ij/). Statistical analysis was conducted using Excel 2003. The same protein lysates were generated for western blot analysis

as shown in Figs. 1B and 2A thus we used the same β-actin as loading control for statistical analysis.

2.4. Immunofluorescence

Immunofluorescence staining was performed as described previously [12]. Briefly, 1 × 0.4 cm strips of skin were fixed, dehydrated and embedded in paraffin wax and sectioned onto slides. The slides were deparaffinized in xylene, rehydrated and treated for antigen unmasking. After blocking with 2% BSA/PBS, slides were incubated with primary antibodies overnight at 4 °C followed by incubation with Alexa Fluor 594 conjugated anti-mouse secondary antibody for 30 min at RT. After removal of antibodies, slides were rinsed with PBS and mounted with mounting medium containing DAPI (H-1200, Vector, Burlingame, CA). Fluorescence was immediately recorded on an Olympus EX51 microscope.

2.5. Immunohistochemistry

Immunohistochemistry staining of p-Yap was performed using EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC Kit (ab94710, Abcam, Cambridge, MA) according to manufacturer's standard protocol.

2.6. Real-time PCR

RNA isolation and cDNA synthesis was performed as described previously [12]. Real-time PCR was performed in iQ™ SYBR® Green PCR Master Mix (170-8880, Bio-Rad, Hercules, CA) using primers obtained from Invitrogen as listed in Table S1. Comparative ΔΔCt method was used in the quantification of mRNA expression.

2.7. Statistical analysis

Statistical analysis was performed using the Student's *t*-test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Arsenic up-regulates Hippo signaling in murine skin

Earlier, we showed that arsenic induces inflammatory response in the skin of SKH-1 mice which may be associated with some the pathobiological alterations reported in arsenic-exposed populations [12]. Here, we show that arsenic induces hyperkeratosis and disorganization of epidermal keratinocytes associated with hyperproliferative phenotype at least in some focal areas of the epidermis. In this regard, disrupted architecture of the basal layer of the epidermis was observed indicating the disruption of tight/adherens junctions formation (Fig. 1A).

Canonical Hippo signaling pathway regulates organ size through a kinase cascade involving Mst1/2 that phosphorylates and activates Lats1/2. Activated Lats1/2 then inactivates the transcription co-activator Yap in a phosphorylation-dependent manner to inhibit proliferation and enhance apoptosis [13]. However, in this study, the WB analysis revealed a dose-dependent increase in Mst1 but no significant changes in p-Mst1 (Fig. 1B and C). The levels of p-LATS1 were significantly increased by arsenic

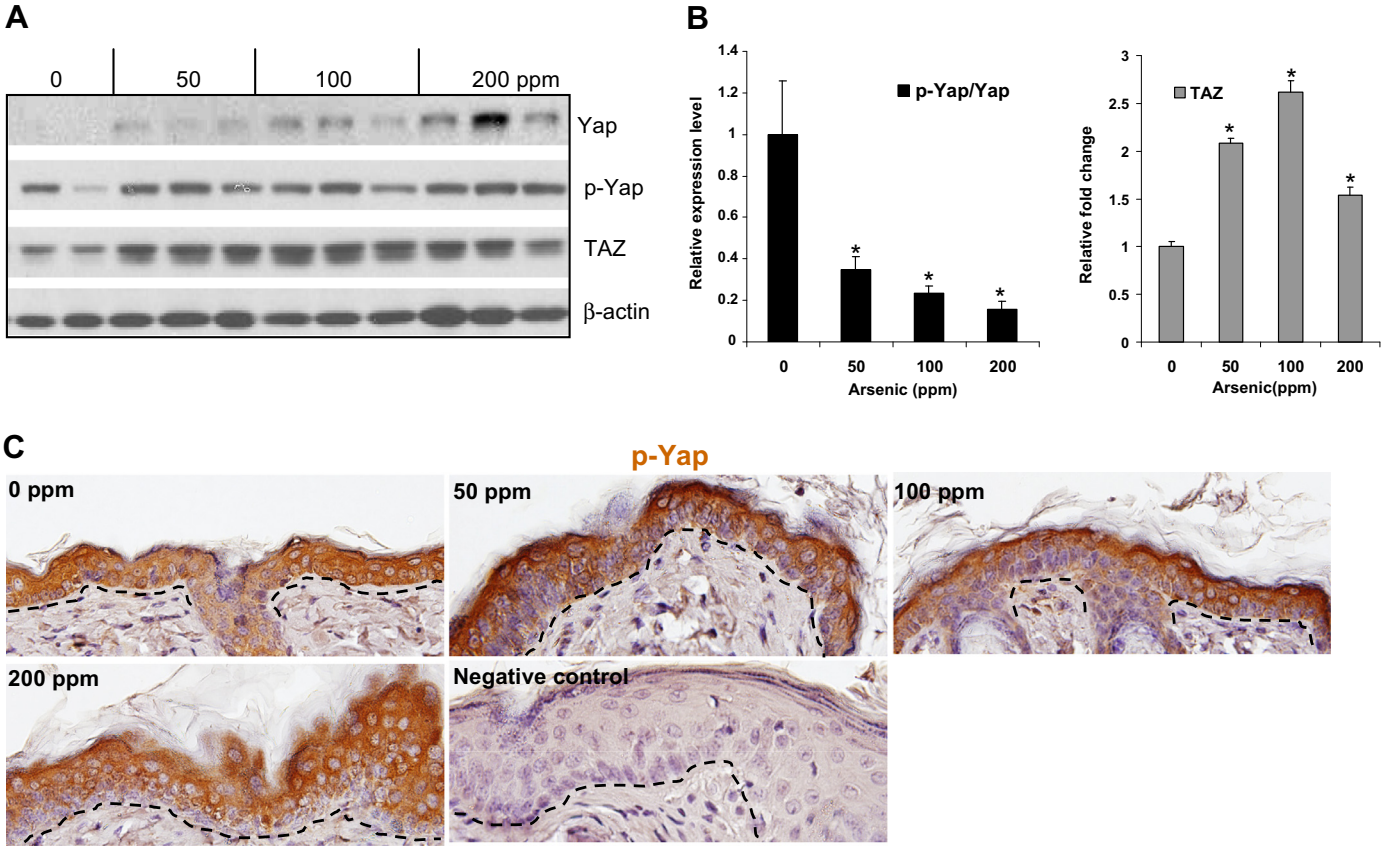

Fig. 2. Effects of Arsenic on p-Yap in murine skin. (A) & (B) Western blot and statistical analysis showing the expression of p-Yap, Yap, and TAZ in the skin of SKH-1 mice. *indicates $p < 0.05$ when compared to control. Bars represent mean ± SEM ($n = 3$). (C) Immunohistochemistry staining showing decreased expression of p-Yap expression in the skin of arsenic-treated animals. Note that the basal layer of epidermis manifested a dose dependent down-regulation of p-Yap staining. Each picture is representative of three independent skin samples. The dashed lines delineate the boarder of epidermis and dermis.

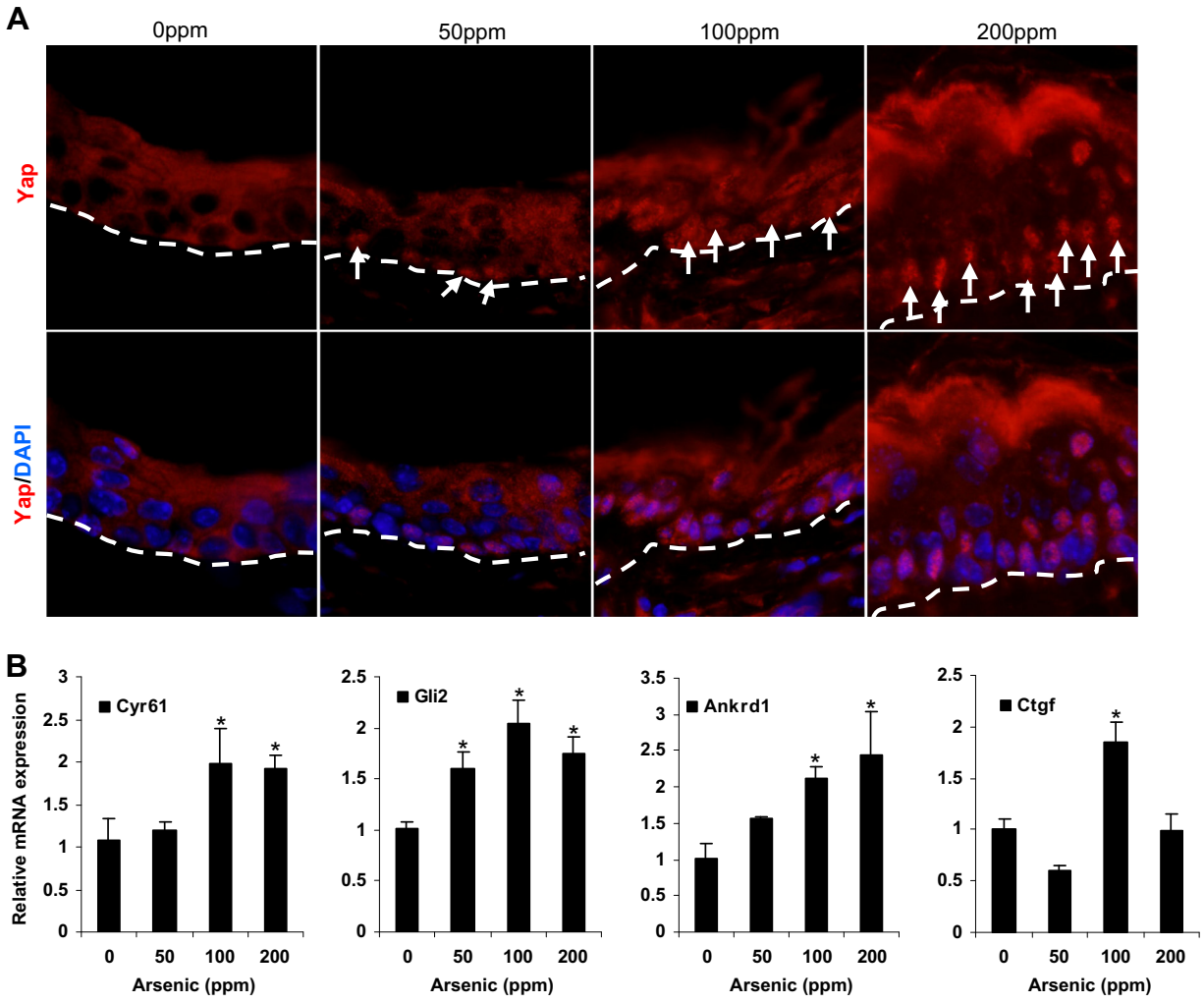

Fig. 3. Arsenic enhances Yap-dependent transcription in murine skin. (A) Immunofluorescence staining showing the expression of Yap (red) in the skin of SKH-1 mice. Nuclear localization of Yap was absent in control skin whereas a dose-dependent induction was observed in 50 ppm, 100 ppm and 200 ppm sodium arsenite treated animals. The dashed lines represent the boarder of epidermis and dermis. DAPI was used to stain the nucleus. White arrows indicate the co-localization of Yap and DAPI staining. Each picture is representative of three independent skin samples. (B) Real-time PCR showing the relative expression levels of Yap-dependent genes, Cyr61, Gli2, Ankrd1 and Ctgf. *indicates $p < 0.05$ when compared to control. Bars represent mean ± SEM ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 1B–D). The Hippo pathway requires Sav1 for activation of MST1 and the consequent activation of LATS1/2 [14]. Consistently, the expression of Sav1 was significantly upregulated in the epidermis of arsenic-treated mice (Fig. 1B and C). Similarly, another important Hippo signaling regulatory protein MOB1 which was shown to bind with LATS1/2 thereby stimulating its kinase activity was also increased by arsenic (Fig. 1D).

3.2. Arsenic induces Yap activation independent of Hippo signaling pathway

Despite the upregulation of Hippo signaling, arsenic-treated skin showed a dose-dependent increase in the expression of Yap and a decrease in the ratio of p-Yap/Yap which suggests a reduced accumulation of phosphorylated Yap in the skin (Fig. 2A and B). Consistently, the homolog of Yap, TAZ was also up-regulated significantly in a dose-dependent manner (Fig. 2A and B). Immunohistochemical analysis confirmed a decrease in p-Yap expression mainly in the basal layer of epidermis of arsenic-treated animals (Fig. 2C). Recently, it has been shown that Yap acts downstream of adherens junction protein αCatenin to control proliferation in the skin [8]. Therefore, we also examined the expression of αCatenin in the skin. A dramatic increase of αCatenin was observed in arsenic-treated skin (Fig. 1B and C). These data indicates that αCatenin fails to recruit Yap to adherens junction and maintain it in its transcriptional inactive form p-Yap. The activation of Yap is also consistent with the induction of epidermal hyperplasia in arsenic-exposed mice (Fig. 1A).

3.3. Arsenic induces Yap-dependent transcription activity

The phosphorylation status of Yap is a critical determinant of its sub-cellular localization and transcriptional activity [14]. Immunofluorescence analysis revealed that Yap was mainly localized in the cytoplasm in control skin (Fig. 3A). However, an increase in the nuclear localization of Yap was observed in arsenic-treated skin in a dose-dependent manner (Fig. 3A). These changes were more prominent in the basal layer of the epidermis. However, at the higher dose of arsenic, nuclear Yap expression could be observed in both basal and superabasal layers (Fig. 3A). Transcriptional activity of nuclear Yap is also evident. Enhanced mRNA levels of

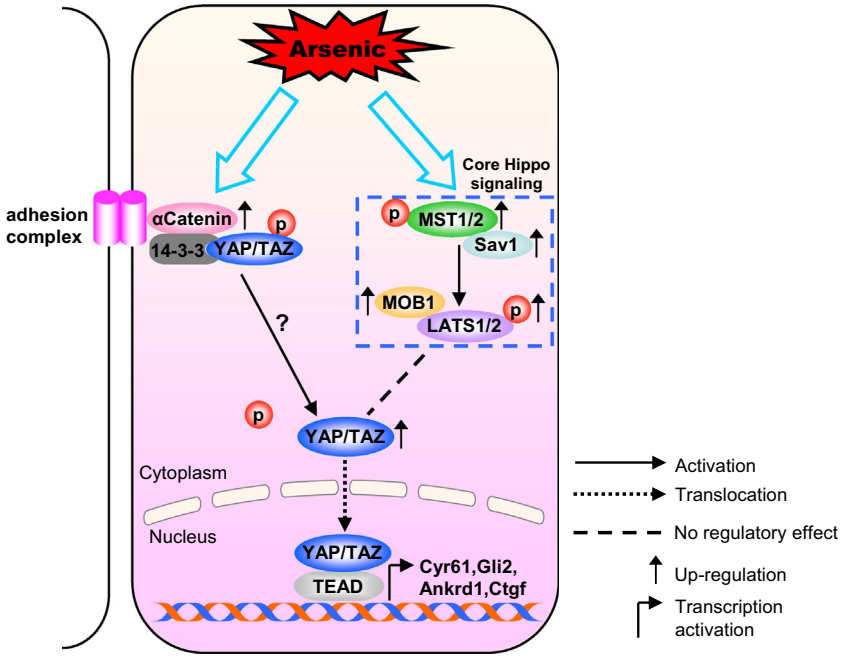

Fig. 4. Shematic flow diagram showing arsenic-mediated Hippo signaling and Yap activity in the murine skin. Sub-chronic exposure to arsenic leads to up-regulation of multiple Hippo signaling components including MST1/2, Sav1, p-LATS1 and MOB1. This is in addition to enhanced αCatenin expression in the murine epidermis. Nevertheless, arsenic activates Yap independent of these upstream events and consequently Yap migrates into the nucleus and induces transcription of its target genes including Cry61, Gli2, Ankrd1 and Ctgf.

Yap/TAZ-TEAD target genes such as Cyr61, Gli2, Ankrd1, and Ctgf was observed (Fig. 3B).

4. Discussion

The most common skin lesions in arsenic-exposed population are hyperpigmentation and hyperkeratosis prior to development of Bowen's disease (carcinoma in situ), BCC and SCC [4]. Oxidative stress, chromosomal abnormality, altered growth factors provide a basis for some of these alterations [15,16]. However, the exact mechanism by which arsenic induces the patho-biological changes remains poorly understood. Previously, we have shown that induction of unfolded protein response (UPR) signaling pathway is associated with the pathogenesis of cutaneous inflammation [12]. Here, we show that arsenic up-regulates multiple components of Hippo signaling pathway including MST1, Sav1, p-LATS1 and MOB1. The exact pathobiological importance of these changes at this stage is unpredictable. It is clear from our results that activation of Hippo signaling kinases does not inhibit Yap through its phosphorylation. On the contrary, we observed up-regulation of Yap by its nuclear localization and subsequent transcriptional activation. These data suggested that arsenic may regulate Yap independent of canonical Hippo signaling pathway in the skin. In this regard, Schlegelmilch et al. reported that abrogating the expression of MST1/2 and LATS1/2 did not change the pattern of Yap phosphorylation or its transcription activity in human keratinocytes. Instead, they identified αCatenin as a novel upstream regulator which can independently regulate Yap activity in the skin likely through its binding with Yap at the plasma membrane [8]. Interestingly, arsenic augments cutaneous expression of αCatenin but without regulating the adherens junction formation by associating with p-Yap. Cytoplasmic p-Yap forms complex with 14-3-3 and αCatenin leading to development of adherens junctions [8]. Recently, 14-3-3 was shown to play a key role in retaining epithelial polarity through the assembly of adherens and tight junctions [17,18]. Thus, the disruption of junctions in arsenic-treated skin might be attributed to dephosphorylation of Yap which triggers the disassembly of p-Yap/14-3-3/αCatenin complex at adherens junctions. In addition, cytoplasmic p-Yap is shown to be important in maintaining tight junction by preventing Angiomotin-like 1 (AMOTL1), an important component of tight junction formation, from degradation [19,20]. Interestingly, Yap is a dual function protein acting like β-catenin, while it regulates the formation of tight/adherens junctions when phosphorylated, the unphosphorylated form regulates epithelial proliferation by its transcriptional activity. One of its transcriptional target genes is Gli2 which may be involved in the pathogenesis of arsenic-induced BCC as reported in humans [21,22]. However, this remains to be confirmed.

It is likely that arsenic may induce a phosphatase which may dephosphorylate p-Yap leading to its leaching from the 14-3-3 protein which is known to help in retaining p-Yap in the cytoplasm by its sequestration. The likely potential phosphatase candidate in this regard may be PP2A phosphatase [8]. Taken together, we show for the first time that arsenic activates Yap independent of the activation of Hippo signaling and upregulation of αCatenin in murine skin. This leads to transcriptional activation of Yap and consequent trigger of proliferation (Fig. 4). These data provide a tempting novel mechanism through which the carcinogenic effects of arsenic may be mediated. However, further studies are needed to unravel the exact mechanism by which arsenic induces the activation of Yap.

Acknowledgments

This work has been partially supported by funding provided by NIH/NIEHS R21ES017494 and NIH/NIAMS R21AR064595 to M.A.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.008.

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