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Review

Crystallographic mining of ASK1 regulators to unravel the intricate PPI interfaces for the discovery of small molecule

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A R T I C L E   I N F O

A B S T R A C T

Protein seldom performs biological activities in isolation. Understanding the protein–protein interactions' physical rewiring in response to pathological conditions or pathogen infection can help advance our comprehension of disease etiology, progression, and pathogenesis, which allow us to explore the alternate route to control the regulation of key target interactions, timely and effectively. Nonalcoholic steatohepatitis (NASH) is now a global public health problem exacerbated due to the lack of appropriate treatments. The most advanced anti-NASH lead compound (selonsertib) is withdrawn, though it is able to inhibit its target Apoptosis signal-regulating kinase 1 (ASK1) completely, indicating the necessity to explore alternate routes rather than complete inhibition. Understanding the interaction fingerprints of endogenous regulators at the molecular level that underpin disease formation and progression may spur the rationale of designing therapeutic strategies. Based on our analysis and thorough literature survey of the various key regulators and PTMs, the current review emphasizes PPI-based drug discovery's relevance for NASH conditions. The lack of structural detail (interface sites) of ASK1 and its regulators makes it challenging to characterize the PPI interfaces. This review summarizes key regulators interaction fingerprinting of ASK1, which can be explored further to restore the homeostasis from its hyperactive states for therapeutics intervention against NASH.

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1. Introduction

The comprehensive knowledge of the molecular mechanisms underlying disease is vital for accelerating the development of novel therapeutic interventions. In this regard, the protein–protein interaction (PPI) network serves as a basic frame for the change in signaling circuitry that regulates the physiological response as it highly depends on the types of interaction network of regulatory proteins [1]. Biological networks are increasingly being shown to be necessary, if not sufficient, for a greater understanding of these mechanisms. The discovery of disease-specific functional modules in human interactomes can give a more focused understanding of the disease's molecular mechanisms. Hence, a better knowledge of the PPI circuitry might help anticipate gene activity and cellular behavior in response to a variety of signals, especially in physiological and pathological states. To realize the potential of PPI and its capacity to retain the homeostasis condition if one can comprehensively map the key regulators (+ve and/or –ve) and their interactions in qualitative and quantitative ways at the structural, dynamical and residual levels. This microscopic information needs to be explored towards the discovery of therapeutics.

Nonalcoholic steatohepatitis (NASH) which is the more severe form of nonalcoholic fatty liver disease (NAFLD) is the major cause of chronic liver disease, affecting up to 40% of adults and children [2–5]. It has become a serious public health problem in developed nations [2,6]. NASH is the more severe form of the disease marked by hepatocellular death, inflammatory cell accumulation, and fibrosis in various stages, advancing to end-stage liver diseases, specifically cirrhosis and hepatocellular carcinoma [7]. Hepatic steatosis can develop owing to increased absorption of free fatty acids (FFA), increased de novo lipogenesis, reduced fat oxidation, decreased hepatic very-low-density lipoprotein (VLDL) production and impaired autophagy [8–10]. Multiple mediators, including JNK, death receptor 5 (DR5), endoplasmic reticulum (ER) stress, autophagy, and ROS, are involved in pro-apoptotic signaling in NASH [11]. These pathways form a signaling network that either contributes to full apoptosis or allows hepatocytes to survive injury [11]. Recent studies suggest that FFA-induced cell death signals

are detrimental to the NASH progression even though apoptosis is incomplete. This is because pro-inflammatory and pro-fibrotic signals are activated to neighboring parenchymal cells. Inhibiting apoptotic signaling can therefore decrease hepatocyte cell death and inflammation in NASH patients [11]. Moreover, the level of hepatic autophagy declines with age, resulting in lipid deposition in the liver and an elevated prevalence of metabolic syndrome in the elderly. As a result, preventive techniques to enhance autophagic activity may offer a new way to avoid metabolic syndrome and related diseases [12].

Retaining ASK1's normal behavior is crucial to ameliorate the NASH condition, rather than its hyperactive state. To achieve this, an impressive effort is needed to design a drug that inhibits pathological action but retains the physiological activity of ASK1 required to reverse the NASH condition. Therefore, understanding the molecular mechanism behind ASK1 activation in NASH is critical for identifying new treatment targets for this pathway that preserve ASK1's normal function. To achieve this and overcome the problem of the conventional (classic) approach, a fine-tuning of ASK1 is required. Targeting the ASK1 and its regulators could be an alternate route (non-conventional), i.e., a PPI-based drug discovery approach. Generating molecular level information of ASK1's selective PPIs will provide a base to identify the interacting *hotspots* residues and their structural, dynamical, and thermodynamic energetic contribution. Further, this understanding will give a rationale to screen the PPI interface sites that help discover potent small molecules and/or peptide and peptide-based therapeutics for NASH, i.e., reversal of fibrosis. A study has demonstrated the shared hub and bottleneck genes of NASH and Inflammatory bowel diseases (IBD) networks via system biology PPI approach, which can be employed as disease markers and therapeutic targets [13].

Despite numerous ongoing active research, there are no approved viable pharmacological treatments for NASH [7], and attempts to manage the problems that arise from the NASH disease are unsatisfactory [14]. Focusing on the crucial regulator (s) in NASH's pathogenic pathways is essential for developing successful therapies. Since the NASH is caused by a complicated process combining metabolic disorders, uncontrolled chronic inflammation,

and fibrosis [15,16] the optimal therapeutic target(s) should be a signaling factor(s) that mediates these key pathophysiology pathways. There are a number of targets under investigation for the treatment of NASH condition, including, apoptosis signal-regulating kinase 1 (Gilead Sciences: selonsertib), a dual antagonist of C–C chemokine receptor, CCR2 and CCR5 (Allergan: cenicriviroc), a dual agonist of the peroxisome proliferator – activated receptors alpha and delta (PPAR α and δ) (GenFit: elafibrinor) and an agonist of farnesoid X receptor [17] (Intercept Pharmaceuticals: obeticholic acid), though any approved drug against NASH is not reported yet. From an anti-fibrosis point of view, ASK1 appears as the prominent target, however, its druggability at the PPI level needs to be explored in detail.

2. ASK1, a relevant target for NASH?

In NASH, ASK1, otherwise called MAP3K5, is a major facilitator and therapeutic target [7,18–20]. Hyperactive ASK1 signaling has been recognized as a molecular signature in the livers of obese and NAFLD individuals [18–20]. ASK1 is regulated by a vast, dynamic multiprotein signalosome complex that may potentially include over 90 documented ASK1-interacting proteins [21]. The ASK signalosome's multimeric core complex, which includes ASK1, ASK2, and 14-3-3 proteins dynamically bind additional binding partners necessary to drive various stress-response signaling processes [21]. ASK1 stimulates the deregulation of lipid and glucose metabolism [22–25] and prompts the inflammatory response in the liver [26,27] via a downstream mitogen-activated protein kinase 38 (p38)– c-Jun N-terminal kinase ½ (JNK1/2) pathway.

Moreover, ASK1 is important not just for regulating innate immunity and maintaining metabolic homeostasis as it is involved in slowing down the course of various cardiometabolic disorders [28]. In hepatocytes, the ASK1 activation is a crucial stage for NASH progression, therefore, it appears as a promising therapeutic target, however, the mechanism of ASK1 hyperactivation in NASH remains unclear. Due to interaction with various endogenous regulators as ASK1 have multidomain architecture its regulators need to be explored as potential therapeutic targets as tuning of these regulators can help to restore homeostatic states. For specific modulation of ASK1, here, we have characterized the discussion into two categories first by targeting the catalytic site which is the conventional route, and second via PPI which is the non-conventional approach.

2.1. ASK1 modulation via targeting catalytic site

There are some molecules reported that target the catalytic site of ASK1 [29,30]. In this route Gilead's selonsertib (GS-4997), a selective inhibitor of ASK1, has been tested as a treatment for NASH in a randomized phase 3 STELLER trial, however, the patient results were not at all encouraging [31]. The withdrawal of selonsertib raises the question of whether there is a problem with selonsertib or whether targeting ASK1's catalytic site is safe to ameliorate the NASH condition? Other than the catalytic site what are the key points which still make ASK1, a pharmaceutically relevant target for NASH?

Selonsertib completely inhibits ASK1's activity by being an ATP competitor for its catalytic kinase domain [26]. Its withdrawal is disappointing as it was safe and effective in inhibiting ASK1; however, it failed to reverse fibrosis or slow disease progression in NASH [32]. The analysis of clinical trials postulates the possible reasons for ASK1's failure such as 1)- According to the liver biopsies, selonsertib reduces hepatic p38 phosphorylation, suggesting the lack of clinical efficacy was not due to a failure to inhibit the target, 2)- The 48-week treatment period with selonsertib was insufficient to reverse progressive fibrosis, 3)- Fibrosis in the individuals investigated in these studies may have been too advanced and not susceptible to regression after therapy with selonsertib, 4)- ASK1 inhibition may be inadequate to affect fibrosis due to redundancy in other pathways that drive hepatic damage and fibrosis in NASH. Therefore, it may also interfere with the physiological function of ASK1 [33], in this way potentially prompting undesirable side effects, 5)- complete inhibition of ASK1 potentially leading to unwanted side effects, and 6)- ASK1 is a widely expressed protein that is important for cell survival, homeostasis, and/or metabolic activities. As a result, selonsertib monotherapy is ineffective.

Another emerging *ray of hope* of small molecules by targeting catalytic site is the discovery of the compound SRT-015 (the seal rock company). This compound is a *best-in-class* inhibitor with demonstrated efficacy in a diet-induced obese mouse model of NASH by alleviating all the key drivers of NASH such as inflammation, liver fibrosis, and hepatic cell death [https://www.seal-rocktx.com/aasld-2020, https://www.sealrocktx.com/aasld-2020-1]. It also decreases plasma AST, ALT, and total cholesterol levels. Selonsertib, on the other hand, displayed either only minor or no hepatic effectiveness in *in-vivo* when exposed to equivalent liver exposures. The SRT-015 is scheduled to reach phase I clinical trials, depending on its substantial efficacy.

2.2. ASK1 modulation via its domain-specific modulation through PPI approaches

Excess or inadequate activation (hyperactive) can disturb homeostasis and induce pathology, due to imbalance, therefore maintaining ASK1 status in a diseased state is crucial. As a result, fine-tuning ASK1 activity toward homeostasis could be a better alternative to cope with the disease rather than complete inhibition. In this regard, clarifying the molecular mechanism behind ASK1 hyperactivation in NASH to find the new therapeutic target(s) of this pathway that preserve the ASK1 normal function or help reverse ASK1 status seems significant clinical importance. The PPI approach is further strengthened due to the failure of Selonsertib which indicated the complete inhibition of the catalytic site, possibly not the solution of NASH improvement and/or anti-fibrosis, led to unraveling the PPI as a potential route for NASH. The meticulous tuning of the activity of ASK1 to mitigate and suppress these pathologies and slow down the progression of NASH is suggested earlier as well [7,18,19]. The PPI routes for modulation can work in two ways: a1. via its endogenous positive regulators (TRAF2/6, USP9X, TRIM48, and TNFAIP3) and negative regulators (TRX, 14-3-3, CFLAR CREG, Roquin-2, and DKK3) and, a2. by targeting the most influential regulators for normal physiology of ASK1 and exploring their +ve and −ve regulators to maintain its homeostasis. For example, we find to explore 14-3-3 and its regulators as a novel approach by identifying a motif from its binding partners. This approach has potential though it is challenging and advanced methods are needed for better resolution via exploring the protein–protein interaction approaches to lead the path. One such example is the complex between ASK1 and TRAF6. The TRAF6-mediated Lys6-linked ASK1 ubiquitination accelerates the dissociation of thioredoxin from ASK1 as well as ASK1 N-terminal dimerization, resulting in the enhanced activation of ASK1- JNK1/2-p38 signaling cascade in hepatocytes [34]. TRAF6-mediated Lys6-linked ASK1 polyubiquitination is a significant driver of pro-inflammatory and pro-fibrogenic responses in NASH, as well as a mechanism underpinning ASK1 activation in hepatocytes [34]. Though targeting PTM sites for therapeutics discovery is a challenge, blocking Lys6-linked ASK1 polyubiquitination could be a promising therapeutic target for NASH therapeutics.

Another hallmark in NASH is the activation of autophagy by ASK1 which promotes anti-inflammatory pathways [35]. The

impaired autophagy increases the lipid droplet accumulation and rises to steatosis and liver fibrosis [35–39]. The Autophagy induction seems beneficial in NASH, and since it is also PPI driven, this approach appears as a possible most likely approach to modulate the hyperactive state of ASK1.

3. ASK1 characterization at structural and PPI modulation drive for NASH therapeutics

3.1. Structural multi-domain architecture of ASK1, a challenge for the PPI approach

Human ASK1 protein is of 1374 amino acids sequence length and consists of three major domains, which include the N-terminal TRX-binding domain (TBD), the central regulatory region (CRR) containing the TRAF binding region as well as serine/threonine kinase domain aka catalytic domain (CD) and C-terminus contains a coiled-coil (CC) region (Fig. 1A). Only limited crystallographic information such as CRR (residue 269–658), CD (residue 659–951), and a small 14-3-3 binding motif (residue 963–970) of the ASK1 is available (Fig. 1A). The CRR structure contains 14 helices forming the seven tetratricopeptide repeats and a pleckstrin homology domain [40]. Moreover, the CD structure displayed

the typical kinase fold constituting a small lobe (residue 670–757) with five β sheets and α helix and a large C-terminal lobe (residue 761–940) comprising mainly the α helices (Fig. 1A). Additionally, for more detailed structural architecture and active site information of CRR and CD, readers can refer to the articles [40] and [40,41], respectively. However, the structural segments from residue 1–268 (required for TRX and TNFAIP3 binding) and residue 971–1374 (known for binding with USP9X and PRMT1) are not reported yet (Fig. 1A). Since the structure is the blueprint of the function, it is required to solve the structures of these zones to comprehend molecular mechanism and PPI interface with its main regulators such as TRX, TNFAIP3, USP9X, and PRMT1. Moreover, to execute the structural PPI analysis, the available structural segments of ASK1 and its binding partners are curated (Fig. 2A and 2B).

3.2. The PPI exploration of ASK1 possibly led to explore other therapeutic targets of NASH

There are multiple targets reported against NASH such as FXR, PPAR (α and δ), ACC, CCR2 and CCR5, SGLT2, Acetyl-CoA carboxylase, etc [42]. The interaction of these targets with different ligands are well documented, in which major focus is on the catalytic site.
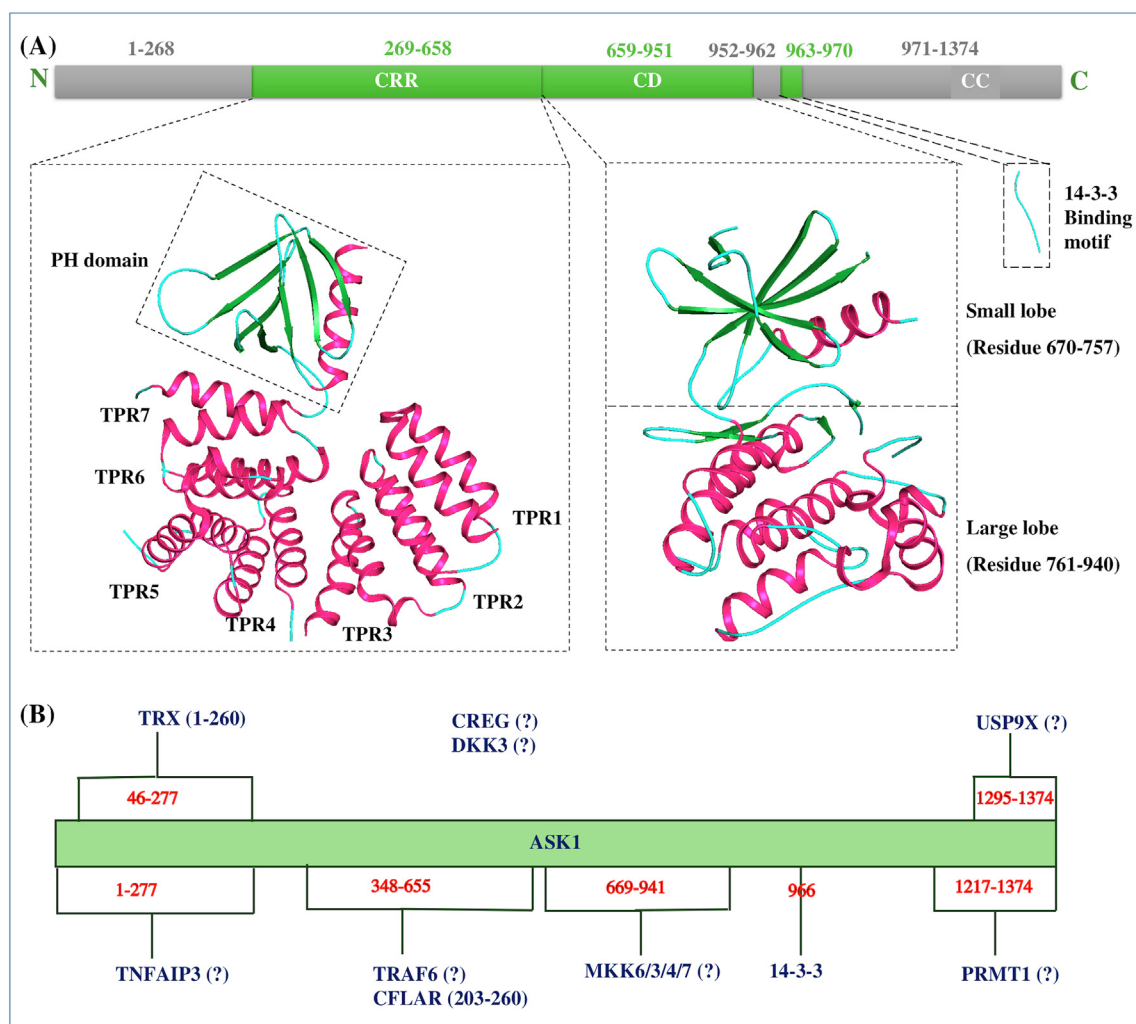
Fig. 1. ASK1-domains and their domain-specific regulators: (A) ASK1's available structural segments (green) and unavailable structural segments (gray) are depicted schematically. (B) ASK1 partner's tentative binding position at ASK1 (red) and ASK1's tentative binding location at its partner (blue), the unavailable information were depicted by the question mark (?). The 14-3-3 is the only partner of ASK1 whose interface residue interaction information is known (refer to Figs. 4 and 5). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

UniProt ID	Gene Name	AA Sequence length	Available segment 1	Available segment 2	Available segment 3	Available segment 4
Q99683	ASK1	1374	269-658	659-977		
P10599	Trx	105	1-105			
P63104	14-3-3 zeta	245	1-245			
Q9Y4K3	TRAF6	522	50-211	346-504		
O15519	CFLAR	480	62-73	209-480		
O75629	CREG	220	49-220			
Q9UBP4	DKK3	350				
P21580	TNFAIP3	790	1-370	381-416	592-635	757-790
Q93008	USP9X	2570	880-970	1551-1970		
Q9HBD1	Roquin-2/RC3H2	1191	86-404	171-325		
Q8IWZ4	TRIM48	208				
Q99873	PRMT1	371	1-371			
Q96J02	ITCH	903	1-155	246-270	328-392	436-903
P46734	MKK3	347				
P45985	MKK4	399	80-399			
P52564	MKK6	334	1-334			
P52564	MKK7	419	37-48	101-419		
P45983	JNK1	427	1-379			
Q15759	P38	364	1-364			

Fig. 2. Curation of structural domains of ASK1: (A) Available structural segments of ASK1 and its binding partner are shown in blue, while unavailable structural segments are shown in yellow (unavailable segments are given the numbers with prime whereas available segments numbers are without prime). (B) information about the ASK1 segments that are available, as well as their binding partners. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

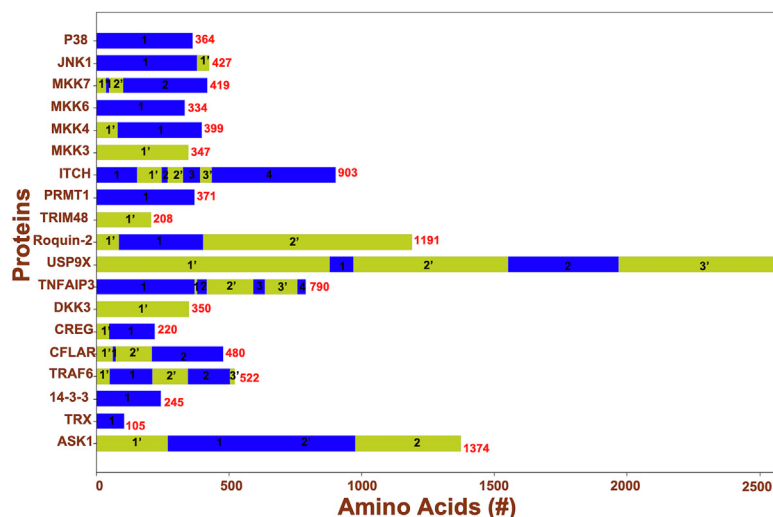However, for most of the targets the PPI approach is unexplored. For example, FXR (the most established anti-NASH target),in which the substrate in form of co-activator/co-repressor is modulating their function via PPI, however, their role and characterization to discover small molecules is not well explored. Furthermore, the post-translational modifications that worked through the PPI manner are also not explored for PPI modulation. One of the reasons for less exploration is the lack of PPI resolution and characterization of the interface site at the residue level. Moreover, with respect to other targets, the rationale to explore ASK1 through PPI ways is due to its multi-domain architecture which is known for its involvement in multiple biological pathways. Another possibility for its PPI exploration is for selective/specific modulation or binding partner-specific modulation which allows drug discovery pipeline towards specificity. Henceforth domain-specific modulation via the PPI approach seems a potential approach rather than complete inhibition as it can induce the off-target effects which are visible nicely and could be a possible region of failure of selonsertib.

4. ASK1 to restore the homeostasis via type and regulators of its modulation for NASH

4.1. ASK1 modulation via inhibition or activation?

ASK1 inhibition or activation both are reported for the betterment of liver health in animal models [18,43-46], which need to be explored due to contradictions in terms of activity. According to Wang et al. (2017), CFLAR inhibits ASK1 by preventing its dimerization, while Zhang et al. (2018) found that the endogenous suppressor TNFAIP3 inhibits ASK1 by preventing its deubiquitination [43]. The ASK1 inhibition has also been highlighted by Schuster-Gaul et al. (2020) and Loomba et al. (2018) for improving liver health in NASH [45,46]. In contrast to these findings, Challa et al. (2019) demonstrated that ASK1 overexpression (activation) has been shown to improve liver health in NASH conditions [44]. They have reported that ASK1 knockout mice build up a more serious hepatic steatosis, inflammation, and fibrosis. In addition, pharmacological inhibition of ASK1 expanded hepatic lipid aggregation in wild-type mice [44]. Inline, liver-explicit ASK1 overexpression shields mice from developing hepatic steatosis fibrosis [44]. In subjects of lean and obese human livers, expression of ASK1 negatively correlated with liver fat content and NASH scores, however positively with autophagy markers [44]. These observations contradict previous research, posing the complex and nebulous issue of whether ASK1 activation or inhibition would be the therapeutic solution for the NASH disorder. A complete understanding of ASK1 activation/inhibition mechanisms and ASK1-binding proteins that control the ASK1 activity is needed to address the above-mentioned questions.

4.2. Relationship between positive and negative regulators to maintain the homeostasis

The positive and negative regulators' role is to modulate the biological status (like an ON/OFF switch) with an interacting partner to maintain homeostasis. Since one of the disease states is the imbalance of relationship between +ve/−ve regulators, therefore, it is interesting to explore the pathophysiology-specific modulation of drug targets via their regulators. The relationship between regulators and druggable target depends on interactions, interaction residence time, and the cell health status which changes from physiological status to pathophysiological condition. Therefore, exploring the positive/negative regulators is essential to understanding the molecular mechanism of the target. Here, the positive and negative endogenous regulators of the ASK1 are responsible to maintains the normal functioning of ASK1 and maintaining the homeostasis, therefore, the endogenous regulators of ASK1 are explored as potential therapeutic targets, as tuning of these regulators can help in restoring back towards homeostatic states of ASK1 in particular at diseased conditions on which the positive regulators are either downregulated or inactive, therefore, fail to maintain the homeostasis. Similarly, the negative regulators are either overexpressed or hyperactive (either copy number or concentration), hampering homeostasis. Therefore, enhancing the positive regulators and perturbing the negative regulators in diseased conditions could be a hallmark to approach therapeutic intervention. In this regard, exploring pivotal regulators can accelerate the pace of small molecular discovery. The imbalance expression, defective activity (hyper or hypo) or defective inactivity in either positive or negative regulators triggers the disease conditions. As a result, sustaining constant optimum function requires homeostatic regulation of these regulators (both positive and negative).

4.3. Regulation of ASK1 status: A possible way to restore the homeostasis

Designing a drug that inhibits pathological action but retains the physiological activity of ASK1 will be ideal for the reversal of the NASH condition, which seems unachievable by direct inhibition of ASK1 [29]. Therefore, understanding the molecular mechanism underlying the transition of ASK1 from its diseased hyperactive state to normal status in NASH is crucial. Finding new therapeutic targets associated with this pathway that preserve ASK1's normal function (at homeostasis) may pave the way toward reversing fibrosis. In general, the endogenous homeostasis in any path is retained by allosteric regulation of protein that plays a significant role in the physiological cell function, biochemical pathways, and signal transduction pathways [47–49]. Knowledge of these regulators would benefit disease understanding [47–49]. Since the clinical trial studies have already confirmed that complete inhibition of ASK1 kinase activity interferes with the normal physiological function of ASK1 [29], there is an unmet requirement for alternative orthosteric or allosteric modulators to retain the normal physiological function of ASK1.

5. PPI: A challenging route to modulate ASK1

The PPIs play a significant role in life processes, making them one of the most attractive drug discovery targets. Targeted regulation of PPIs has a vast potential in human physiology, with estimates ranging from 300,000 to 650,000 [50]. PPIs function in complex networks that are highly reliant on the cellular environment and can be significantly changed in disease states. The studies have reported that abnormal PPIs are associated with numerous diseases, including cancer, infectious diseases, metabolic disorders, and neurodegenerative diseases [51–53]. Hence, targeting PPIs is a deserving route for therapeutic discovery, though it is challenging due to the ineffectiveness of the classical medicinal chemistry approaches, lack of ligands references, lack of guidance rules, the significant substantial barrier, and lack of high-resolution PPI proteins structures etc. [54,55]. Also, the PPI interface is poorly characterized as the binding sites are mostly shallow and solvent-exposed, and their architecture is unknown. However, significant conceptual and technical advancements in molecular cell biology, biochemistry, and biophysical approaches have gradually revolutionized PPIs in recent years [56]. Indeed, just a few PPI modulators have made it into clinical trials. It is also encouraging that some of them have been approved for marketing, demonstrating that the modulators targeting PPIs have wide-ranging prospects [57–62]. Since the structural wealth of interacting proteins is a prerequisite for drug design, however, the availability of high-resolution interacting protein structures as PPIs are minimal. Therefore, more structural information resources are needed to be added for experimentally validated PPIs.

Inhibitors and stabilizers are the two significant ways to modulate the PPIs [62,63]. Peptides, which can bind at the interface, have shown higher affinity and specificity than traditional small molecule inhibitors and make binding easier with target proteins. Albeit having issues such as instability under *in-vivo* conditions and poor membrane permeability, many peptides have been approved by FDA [64]. To improve the stability of the peptide, chemical modifications can be applied, which prevent rapid degradation [62]. Next, to solve the poor membrane permeability problem of the peptide, a class of short peptides has been discovered that can penetrate the biomembrane and intervene in transmembrane transduction of the macromolecular substances [65,66]. This carries critical advancement to intracellular peptides' development, which enhances the possibility to explore the PPIs as therapeutics.

Another noteworthy advancement has been made in developing antibodies that regulate the PPIs, such as in immunotherapy, the monoclonal antibodies that regulate PD-1/PD-L1 interaction [67–69]. Yet, due to associated high research cost, instability as well as potential severe immunogenic undesirable side effects, increasingly more consideration has been drawn to the small molecular inhibitors and peptides, mainly the small molecular inhibitors as it is associated with lower research costs, various preparations, oral administration, and better penetration to the tumor microenvironment.

6. ASK1 regulators: A potential candidate to explore for therapeutics via PPI approaches

6.1. Regulatory structure of ASK1

The ASK1 structure is defined by long N- and C-terminal sequences and a central serine/threonine kinase domain. The phosphorylation of the crucial residue threonine 838 (T838) in the activation loop of the kinase domain upregulates the kinase activity by inducing the conformational changes at the N- and C-terminus [70]. In addition to the conformational changes of the kinase domain, the activity of ASK1 is also regulated by an auto-regulatory scaffolding system [40]. The study has also reported that the central regulatory region (CRR), which lies between the kinase domain (CD) and TRX binding domain (TBD) of ASK1, mediates the regulation of ASK1 activity. Moreover, the central regulatory region primes the phosphorylation of MKK6 which promotes the ASK1-MKK6 signaling pathway.

6.2. Role of ROS in ASK1 regulatory mechanism

Reactive oxygen species (ROS) are produced inside the cells by cellular metabolic processes such as cell survival, stressor reactions, and inflammation [71,72]. Moreover, low-level generations of ROS are required to support physiological functions, such as proliferation, host defence, signal transduction, and gene expression [73]. Cells maintain the balance between generation and clearance of ROS under physiological conditions, as eukaryotic cells have numerous anti-oxidative defence mechanisms, together with anti-oxidants and enzymes. However, when cell ROS overproduction overwhelms the inherent antioxidant capacity, then there is an occurrence of oxidative stress, which may further damage the biomolecules of normal cells and tissues [73]. On top of that, an imbalance in the ROS physiological mechanism leads to numerous pathophysiological conditions [74–78]. ASK1 activation after ROS generation mediates an essential role in cellular responses under oxidative stress conditions [79]. Under oxidative stress conditions, ASK1 activity is regulated by several positive (TRAF2/6, USP9X, TRIM48, and TNFAIP3) and negative regulators (TRX, 14-3-3, CFLAR CREG, Roquin-2, and DKK3). The PTMs such as phosphorylation, methylation, ubiquitination, and nitrosylation are also reported to be critically involved in the regulation of ASK1 (Fig. 3).

6.3. Negative regulators of ASK1 in oxidative stress conditions

6.3.1. Interaction with 14-3-3

Among different −ve regulators, the 14-3-3 regulatory information and the interface information with ASK1 (in the form of residue peptide 963–970) are reported, which can facilitate to set of the protein–protein/protein-peptide interaction benchmark to understand the interface (Fig. 4). The 14-3-3 is an adapter protein that regulates a large range of general and specialized signaling pathways such as stress responses, cell-cycle control, metabolism, apoptosis, protein trafficking, signal transduction, transcription,
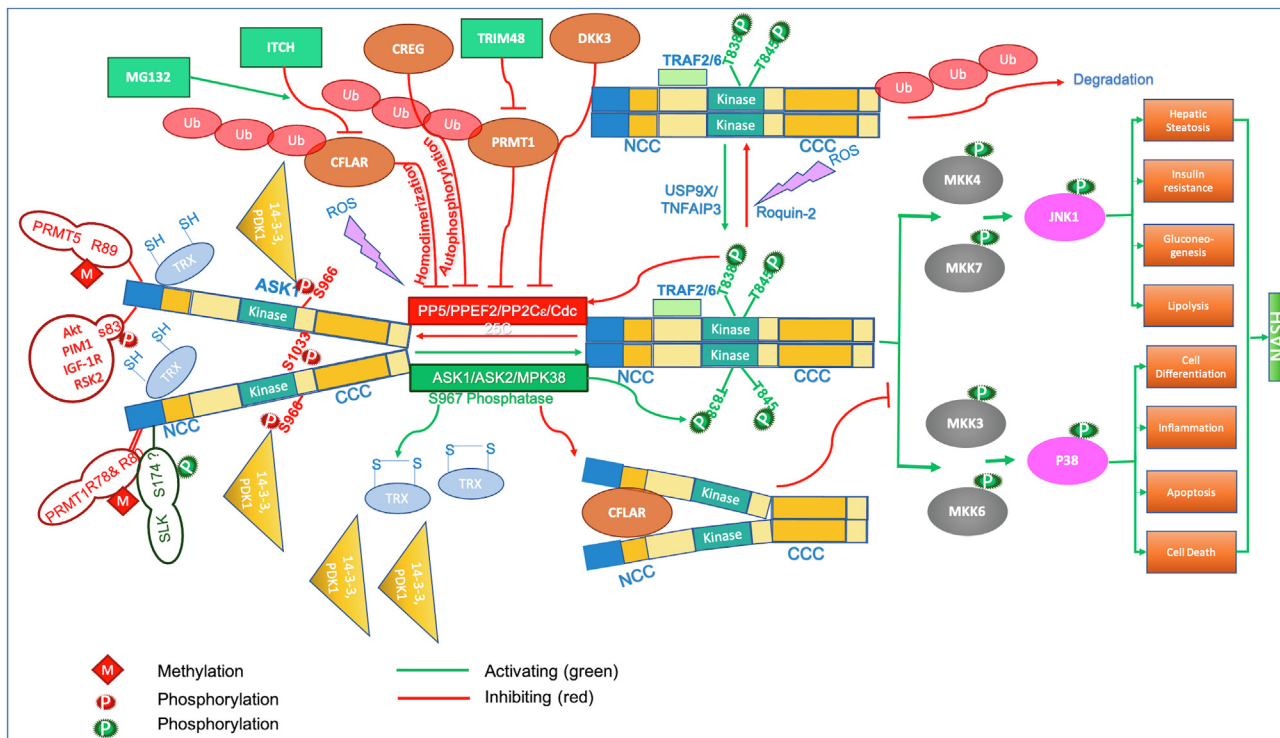
Fig. 3. Mechanistic regulation of ASK1 in NASH: In oxidative stress, ASK1 activity is regulated by several positive (TRAF2/6, USP9X, TRIM48, and TNFAIP3) or negative regulators (TRX, 14-3-3, CFLAR CREG, Roquin-2, and DKK3) either by PTMs such as phosphorylation, dephosphorylation, ubiquitination, deubiquitination, and methylation or by inducing conformational changes. The TRX binds to the N-terminal of ASK1 and keeps it inactive. However, TRX dissociation from ASK1 by ROS in oxidative stress conditions shifts the ASK1 towards an active state by reciprocally recruiting the TRAF2 and TRAF6. 14-3-3 binds to phosphorylated serine (S966) of ASK1 and inactivates the ASK1. Though, oxidative stress of ROS dephosphorylates the site that leads to the dissociation of 14-3-3 from ASK1, which in turn switches the ASK1 in an active state. CFLAR negatively regulates ASK1 by blocking the N-terminus-mediated dimerization of ASK1. Moreover, in oxidative stress conditions, ITCH mediated the CFLAR degradation by ubiquitinating it and activating the ASK1. CREG, DKK3, and PRMT1 are another negative regulator of ASK1 that shifts the ASK1 towards an inactive state. Even so, TRIM48 activates the ASK1 signalosome by promoting d degradation of PRMT1. Roquin-2 mediates the ASK1 proteasomal degradation by C-terminal ubiquitination of ASK1; however, this process is reversed by the USP9X or TNFAIP3. Activated ASK1 regulates downstream signaling using two main pathways. First, the ASK1-MKK4/MKK7-JNK1 pathway, and second, the ASK1-MKK3/MKK6-P38 pathway.
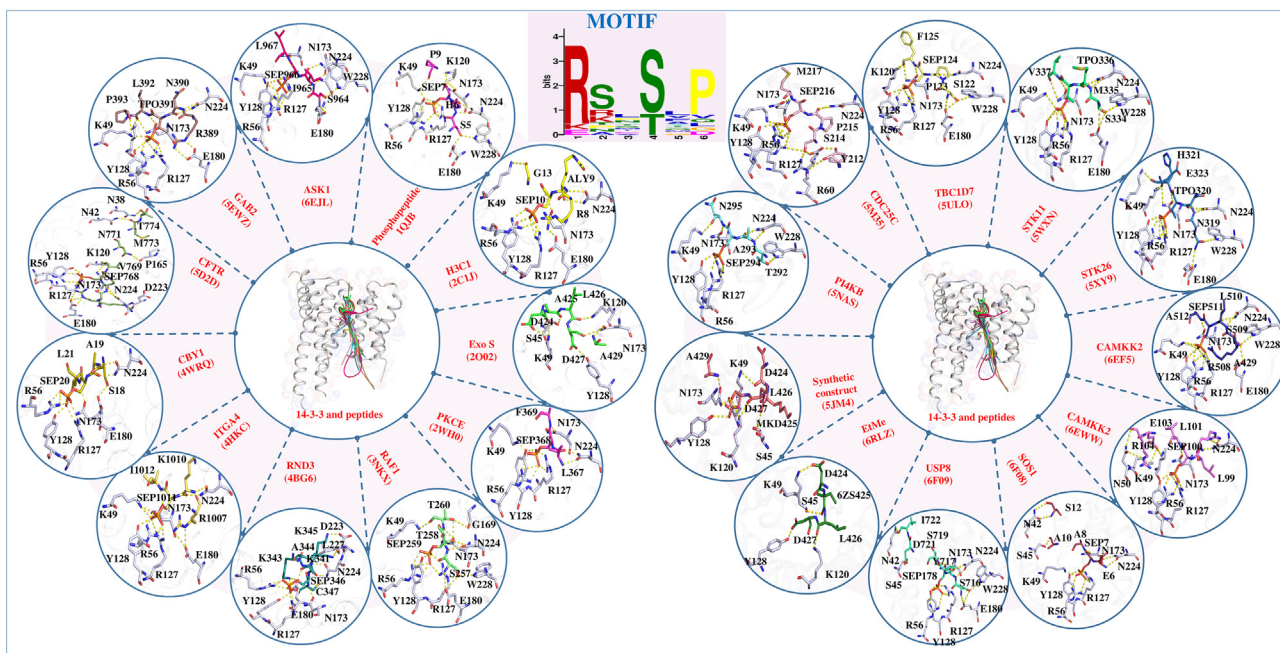
Fig. 4. Residual mapping of 14-3-3 regulators: 14-3-3 and its peptide binding partners are shown schematically. The complex's corresponding PDB-IDs and gene names are written in red. The motif generated from all of 14-3-3's partner peptides is shown in the center. The protein structure was depicted as a cartoon, while the binding interface residues were depicted in a stick format. A zoomed version of the interface residues was also illustrated to have a clearer vision. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and malignant transformation [80]. It was reported that 14-3-3 displayed the differential expression in the liver of NASH patients [81], which suggests its role in NASH pathogenesis [81]. The 14-314-3-3 interacts with many biological partners, generally by identifying a phosphoserine or phosphothreonine motif [82–84]. For ASK1, 14-3-3 binds at phosphorylated serine residue (S966) and inhibits its kinase activity, probably by blocking the access or altering the conformation of the ASK1 active site [85,86].

When levels of ROS are high, the calcineurin sets off the dephosphorylation of ASK1 at S966, by which it triggers the release of 14-3-3 [87–89] from the complex, in turn, it leads to the activation of ASK1 mediated JNK- and P38 MAPK signaling pathway [85,90]. Conversely, IKK, the core element of the NF-κB transcriptional signaling pathways, promotes the interaction between ASK1 and 14-3-3 by phosphorylating ASK1 at S966 residue. IKK, therefore, serves as a central node at the interface of the signaling pathway for pro-survival NF-κB as well as the path for cellular stress response and apoptosis [90]. The stability of the complex of (ASK1 + 14-3-3) is a subject of multiple regulation modes. Zhou et al. (2009) have shown that SOK-1, a member of the mammalian sterile 20 (Mst) kinase family, react to elevated ROS levels by phosphorylating 14-3-3 at S58, causing 14-3-3 to dissociate from ASK1 and activation of downstream pro-apoptotic pathways [91]. Additionally, the ASK1 + 14-3-3 complex interacts with thioredoxin, which may work along with 14-3-3 to inhibit ASK1 kinase activity [92].

Selenoprotein W (SelW) is an intriguing mechanism of 14-3-3 redox modulation because it may create a redox-regulated covalent connection (Sec-Se-S-Cys bond) between selenocysteine on SelW and exposed cysteine on 14-3-3 [93–95]. This interaction is increased under severe oxidative stress and impairs 14-3-3 binding to ASK1. Therefore, the SelW can thus serve as a redox-regulated sink to interfere with 14-3-3 pro-survival interactions [93–95].

Moreover, the above finding demonstrates that 14-3-3 could be a potential target for inhibiting the ASK1 mediated signaling. Therefore, the discovery of the peptides and/or small molecule inhibitors through peptidomimetics from the complex of ASK1 + 14-3-3 complex interface/s seems to have a rationale for the future perspectives towards the discovery of NASH therapeutics. We further explored the interacting interface residues of reported ASK1 peptide and 14-3-3 to mark the *hot-spot* residues, pivotal in establishing the association of ASK1 + 14-3-3 in terms of binding affinity (Fig. 4). Some key interaction was noticed as the ASK1 phosphoserine (SEP966) residue forms hydrogen bonds with residues K49, R56, R127, and Y128 of 14-3-3. In addition, other ASK1 residues such as S964, I965, and L967 are also forming hydrogen bonds with residues N173, E180, N224, and W228 of 14-3-3 (Fig. 4). Moreover, the other interactions in the form of hydrophobic contacts and salt bridges are also identified to enhance the net binding affinity (Fig. 5A). Based on these interaction maps, the key residues can be used to design the peptides
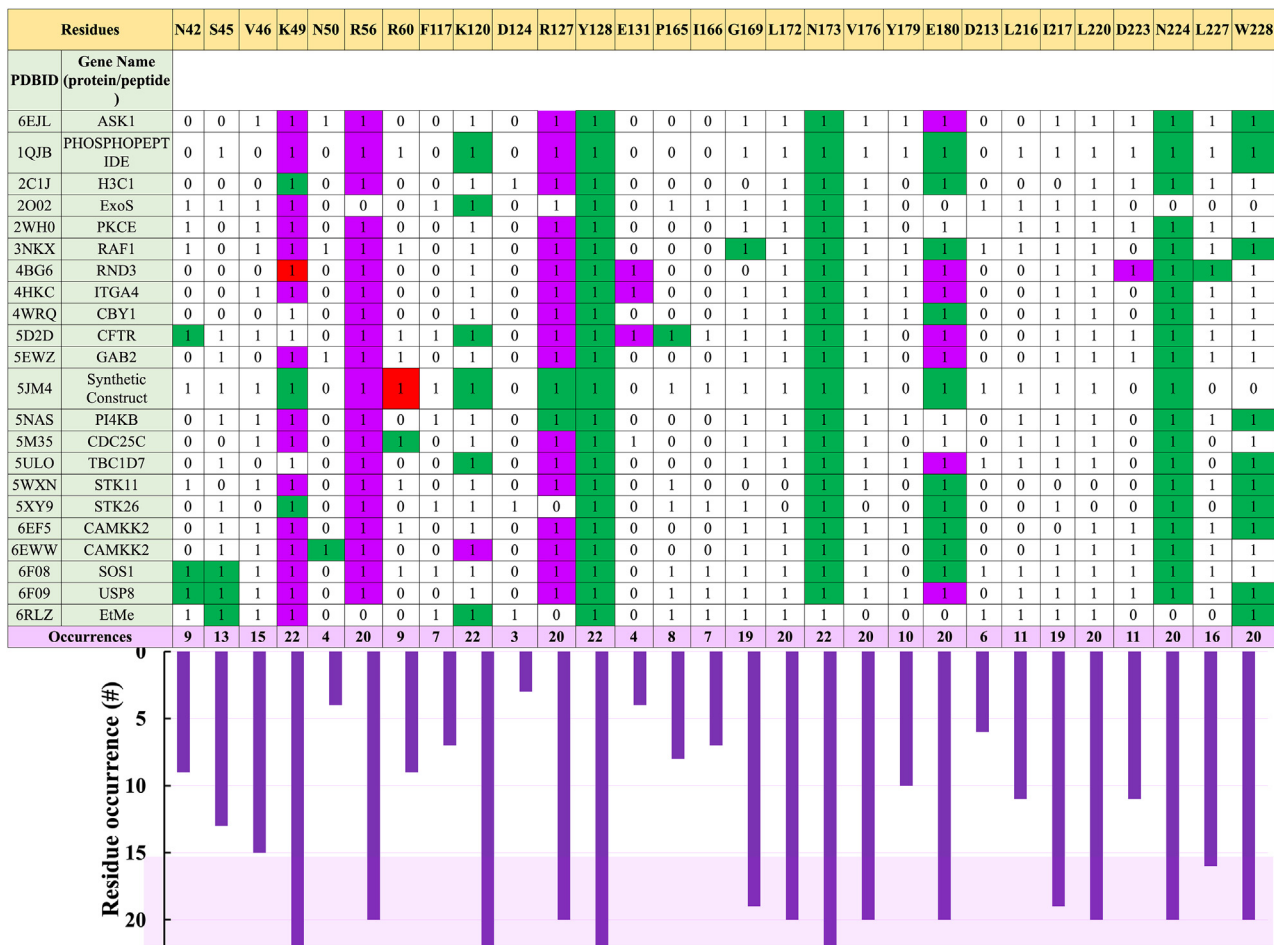
Fig. 5. Interaction fingerprinting of 14-3-3 and its regulators: (A) Occurrences of 14-3-3 residue making the hydrogen bond, salt bridge, and hydrophobic contacts with its binding partners (protein/peptide). The residues with less than 3 occurrences were removed from the table. Residues with/without interaction are shown by 1/0. The interaction types hydrogen bond (HB) in green, HB + salt bridge (SB) in violet, SB only in red and hydrophobic contacts are in white. (B) Occurrences of 14-3-3 residue *hot-spot* primary residues (occurrences > 15) are highlighted in light pink background. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from 14 to 3-3 that can potentially inhibit the hyperactive ASK1 in the diseased state.

At the hyperactive state of ASK1, the endogenous status of −ve regulators such as 14-3-3 is down at protein and gene levels and demonstrated to regulate the course of liver regeneration (Xue *et al.* 2017). In this condition, if we identify the peptides from the 14-3-3 surface which can bind to ASK1 significantly, it can be effective in modulating the hyperactive diseased state. Another PPI approach to target the hyperactive state of ASK1 is to target the endogenous −ve regulators of 14-3-3, which share the common interface with ASK1 (Fig. 4). This way, the peptides designed from the 14-3-3 interface will be able to bind to the surface of ASK1, as well as the same peptide, can bind to −ve regulators of 14-3-3. Consequently, these peptides can inhibit the ASK1 in two ways, first, by direct binding at the ASK1 interface, and secondary, by increasing the endogenous status of 14-3-3 by blocking its −ve regulators. Here, the knowledge of specific +ve/−ve regulators of 14-3-3 can contribute significantly towards therapeutics design.

Since only one crystal of ASK1 and 14-3-3 complexes are reported to date, therefore, the other crystals of 14-3-3 interactors are also explored (Fig. 4). A total of 32 crystals of 14-3-3 complexes (protein–protein/protein-peptide other than ASK1) were extracted from the PDB database to characterize the interface residues. Based on that, we established the benchmark with 14-3-3 peptides. Furthermore, the 22 crystals (out of 32) were selected to characterize the interface residues after excluding the redundant peptide/protein complexes. The interface characterization of 14-3-3 with its binding proteins shares the same interface site as in the case of ASK1, i.e., the crystals revealed a common binding site at the 14-3-3 protein surface (Fig. 4). Furthermore, for each complex of 14-3-3 at the same site, the occurrence of amino acids was characterized, and the most significant residues of 14-3-3 involved in the interactions with its binding partners were monitored (Fig. 5A and 5B). Based on the occurrence, the residues are divided into three categories, namely primary (>15), secondary (>=10 and <= 15) and tertiary (less than 10). The primary residues are considered as *hot-spot* as they are most readily involved in the interaction and evolutionary preferable (based on residues conservation analysis). These analyses revealed an interesting finding that the binding site key residues of 14-3-3 identified from its interaction with ASK1 are almost similar. They share the key residues at the interaction interfaces with other protein/peptide complexes (Figs. 4 and 5). This result also suggests that these *hot spot* residues of 14-3-3 are complementary to the ASK1 peptide and can be used to design novel peptides that could tune the ASK1 activity at its hyperactive state by modulating ASK1 directly and/or by its −ve regulators. Furthermore, the motif analysis using the MEME software [96] from all 22 sequences of 14-3-3 regulators also confirms the key residues. From the identified motif RXXpSXP, in which arginine (R), phosphorylated serine (pS), and proline (P) are highly pronounced in most of the cases (Fig. 4). The same motif structure for 14-3-3 binding was already reported [97], in which the authors used the experimental alanine scanning approach to determine the motif structure. Moreover, an indirect solution focused on a motif derived from 14-3-3 binding partners (or other interactomes) would be a more promising method of modifying ASK1 behavior. Overall, the interface residue analysis of 14-3-3 interactomes is paving the way to design a peptide that might precisely regulate the ASK1 activity.

6.3.2. Interaction with TRX

It's been reported that TRX, in its reducing state, binds to the ASK1 N-terminal region and negatively regulates the activity of ASK1 [98,99]. ROS induces the TRX oxidation in oxidative stress and facilitates its dissociation from ASK1. Dissociation of TRX assists the homo-dimerization of ASK1 via homophilic interaction

of the N-terminal coiled-coil (NCC) region, which activates the ASK1 [99]. Inline, deleted N-terminus mutants of ASK1 (ASK1 ΔN) with lacking TRX binding region showed a rise in basal kinase activity, and the co-expression did not suppress this activity with TRX [98]. ASK1 N-terminal region (residues 46–277) and TRX (residue 1–260) are involved in their interactions [99]. However, the exact interface residue of the ASK1 and TRX complex remains elusive. Here, molecular modelling can pitch in to establish the essential complex to provide structural insight. Therefore, in the absence of ASK1 + TRX co-crystal, the other available crystal complexes of TRX are also explored, assuming that the identified interface residue of TRX could share the same interaction with ASK1 as well. The PPI interface residues of TRX with TRXR1 [100], TXNIP [54,101], and SlrP [54] have been depicted (Fig. 6A). In the case of TRX-SlrP PPIs, there are two types (type 1 and type 2) of interaction interfaces observed, but only type 2 could form the stable connections in the solution [54]. In the interaction map, methionine 74 (M74) residue was common among all three PPI interfaces of TRX (Fig. 6A). This observation suggests that M74 is the key *hot-spot* residue at the PPI interface of TRX protein and could also interact with ASK1. The key residues of TRX can be used for peptide designing from the interaction map, which can bind ASK1 to perturb its active state in the NASH condition.

6.3.3. Interaction with CFLAR-ITCH

ASK1 dimerization and subsequent auto-phosphorylation are necessary for its activation and further downstream activation of the JNK1 pathway. At the N- and C-termini, the NCC (amino acids 297–324) and C-terminal coiled-coil (CCC; amino acids 1239–1295) domains are crucial for ASK1 dimerization. CFLAR inhibits ASK1 activation by interfering with ASK1 dimerization mediated by the N-terminus [18]. This event is mediated by the direct binding of CFLAR to the ASK1 N-terminal fragment (amino acids 278–384) and by the indirect inhibition of TRAF6 recruitment to ASK1 [18]. Taken together, blocking N-terminus-mediated ASK1 dimerization was crucial for the CLFAR-mediated negative regulation of ASK1-JNK1 signaling and for their consequence on the resultant pathology [18]. Wang et al. (2017) have described the downregulation of CFLAR in the human and mice fatty liver and confirmed the downregulation was not because of the transcriptional change of CFLAR mRNA. They have further reported that CFLAR protein expressions are lowered under oxidative stress stimuli (including palmitate, hydrogen peroxide (H2O2), TNF-α, and IL-6) in the cultured primary mouse hepatocyte [18]. The antioxidant introduction (N-acetyl-cysteine (NAC) and enzyme catalase (CAT)) to the mouse model has substantially reversed the ROS-induced downregulation of CFLAR. The group also confirmed that the ubiquitin–proteasome pathway mediates the degradation of CFLAR as proteasome inhibitor MG132 markedly blocked the degradation of CFLAR. Moreover, ITCH [102] was previously identified as an E3 ligase capable of ubiquitinating CFLAR and causing its breakdown in the presence of oxidative stress [18].

CFLAR (FLIP) protein offers a large interface area to its binding partner caspase-8 (C8). Among all the residues at the interface, N447, L467, Q468, H469, and L471 in CFLAR and L455, F453, T452, P451, and N432 in C8 are highly conserved [103]. These residues N447 and L455, L467 and F453, Q468 and T452, H469 and P451, and L471 and N432 form the hydrogen bond which are located at the interface between CFLAR and C8, respectively (Fig. 6D). The conservation of these amino acids indicates that the specificity of the interaction between CFLAR and C8 is likely maintained across different species [103]. In addition, the other hydrogen bonds between residues Q237 and R417, E241 and E430, E242 and Y433, V395 and Q351, and R397 and N349, respectively at the interface, are also crucial in increasing the binding affinity between CFLAR and C8 (Fig. 6D). Moreover, these residues
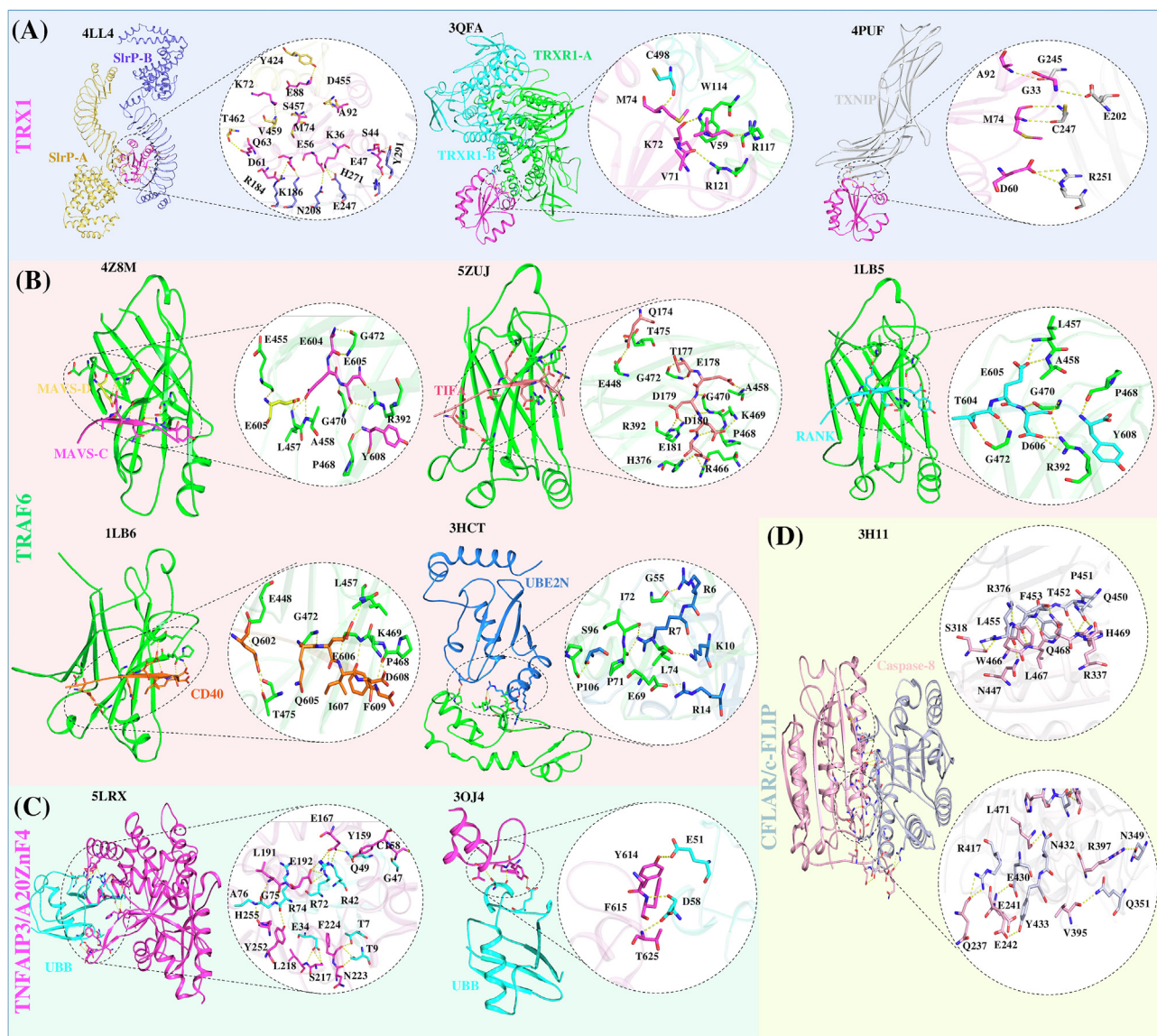
Fig. 6. Exploring the interaction interface of key ASK1 regulators: Schematic representation of (A) TRX1 (magenta color) (B) TRAF6 (green color) (C) TNFAIP3 (or A20ZnF4) (magenta color) and (D) CFLAR (or c-FLIP) (gray color) interaction with their binding partners. The complex's corresponding PDB-IDs are written in black. The protein structure was depicted as a cartoon, while the binding interface residues were depicted in a stick format. A zoomed image of the interface residues was also depicted on the right side of the protein structure to provide a clearer vision. Yellow dotted lines depicted hydrogen bond interactions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are also reported to be involved at the interface of CFLAR (residues 203–260) and ASK1 (residues 384–655) [18]. Therefore, this study unveils the *hot-spot* residues (Q237, E241, E242, N447, L467, Q468, H469, and L471) of CFLAR, which could be involved in the interactions with other proteins as well to modulate the distinct signaling pathways.

6.3.4. Interaction with CREG

Cellular repressor of E1A stimulated genes (CREG) protein plays a significant role in cardiovascular disease phenotypes, inclusive of anti-cardiac ischemia [104], anti-inflammation [105], anti-hypertrophy [106,107], anti-apoptosis (induced by excessive glucose and excessive palmitate [PA]) [108], and anti-fibrosis, basically as a result of its function in promoting differentiation and inhibiting cellular proliferation [109,110]. Interestingly, these cellular processes are evident in hepatic steatosis and metabolic disorders. Moreover, numerous associations between cardiovascular diseases and NAFLD have been discovered [111,112]. Based on gain

and loss of function investigation, it was reported that CREG overexpression ameliorates dietary as well as genetic hepatic steatosis and metabolic disorders. However, deletion of CREG in hepatocytes resulted in exacerbated NAFLD in a JNK1-dependent manner. The *in-vitro* and *in-vivo* studies have illustrated that CREG inhibits the ASK1-JNK1 signaling pathways and, as a result, reduces lipid accumulation and insulin resistance. It's also been reported that CREG dephosphorylates ASK1 by direct physical interaction with it [113], but the exact location of interaction remains elusive. This can also be interesting for computational modelers to unveil the possible interaction sites for better mechanistic understanding.

6.3.5. Interaction with Roquin-2

Roquin-2 (E3 ubiquitin ligase) is involved in the ROS-dependent degradation of ASK1. A point mutation in the ASK1 (ASK1^{KN}) kinase domain illustrates the resistance to oxidative stress (H2O2) induced proteasomal degradation [114]. This suggests that ASK1 kinase activation is necessary for proteasomal degradation. An *in-*

vivo comparative study was performed by ubiquitination of ASK1[KN] and wild-type ASK1 via Roquin-2 overexpression [114]. The study revealed that Roquin-2 overexpression efficiently enhances the K-48 linked polyubiquitination of wild type ASK1 but not of ASK1[KN], suggesting that Roquin-2-dependent ASK1 ubiquitination relies on activation of ASK1 [114]. In a report, it was identified that ubiquitination of ASK1 occurs at C-terminus [115], however, Roquin-2 overexpression only slightly increased the C-terminus ubiquitination [114], which suggests that ubiquitination of ASK1 might occur at multiple lysines over the full length of the protein. Oxidative stress-induced ubiquitination of ASK1 by Roquin-2 inhibits the sustained activation ASK-JNK-P38 MAPK pathway and cell death. Though this ubiquitination is reversed by the USP9X and protects the ASK1 proteasomal degradation, it, in turn, facilitates the ASK1 activity. Hence, the harmony between USP9X and Roquin-2 may add to determine the cell death by temporal regulation of ASK1 activity. Roquin-2 mediated functional regulation of ASK1 activity is evolutionarily conserved [114]. Moreover, a direct interaction interface residue/s information between ASK1 and Roquin-2 is unknown, indeed, that information would be essential for the modulation of ASK1.

6.3.6. Interaction with DKK3

Dickkopf-3 (DKK3) is a secreted glycoprotein belonging to the members of the DKK family [116], and has been broadly examined in the context of cancer, including liver cancer [19]. DKK1, 2, and 4 antagonize Wnt signaling when bound with Wnt co-receptor Lrp5/6 and Kremen proteins [117-121]. In contrast to other DKK family members, DKK3 is not able to bind Lrp and Kremen proteins [117–122] and its receptor remains to be explored [19,123]. Although, the function of DKK3 in the Wnt signaling pathway, as well as hepatic steatosis and associated metabolic disorders, has also been undetermined. DKK3 level is increased in the blood of the aged population. Its expression is increased in prostate basal epithelial cells during cellular senescence [119], suggesting that DKK3 might play a role in ageing-related disorders. An experimental examination reported that Dickkopf-3 (DKK3) expression was remarkably reduced in the liver of NAFLD patients, HFD or genetically induced obese mice, and palmitate stimulated cultured hepatocytes [19]. Based on liver-specific overexpression and knockout study of DKK3, it was shown that DKK3 ameliorates obesity, insulin resistance, and hepatic steatosis. Under oxidative stress, DKK3 binds to the ASK1 and inhibits the ASK1-JNK/p38 pathway [19]. Moreover, a direct interaction interface residue information between ASK1 and DKK3 is elusive, which can add more value towards DKK-specific peptide design.

6.4. Positive regulators of ASK1 in oxidative stress

Apart from negative regulators, positive regulators are also involved in the ASK1 modulation process. In NASH conditions, these binding partners, which are possibly responsible for the hyperactive state of ASK1, need to explore critically. These +ve regulators of ASK1 highly expressed in NASH conditions are supposed to control pathological conditions.

6.4.1. Interaction with TRAF1/2/6

Among the +ve regulators, the TRAFs (TRAF1, TRAF2, TRAF5, and TRAF6) play a role in the ASK1 activation [20,124]. TRAF1 promotes hepatic steatosis by increasing the activation of ASK1-mediated P38/JNK cascades, as demonstrated by the fact that is inhibiting ASK1 eliminated TRAF1's exacerbated effect on insulin deficiency and inflammation and hepatic lipid accumulation [20]. TRAF2 and ASK1 co-expression enhance the homo-oligomerization and activation of ASK1 [125]. Based on the ASK1 deletion mutant study, TRAF2 and TRAF6 have been proposed to bind to the ASK1 amino-

terminal region (residues 384–655) [99]. The binding of TRAF2 and TRAF6 with ASK1 facilitates the ROS status by inducing the ASK1 homophilic interaction via the NCC (N-terminal coiled-coil) region. Moreover, oxidative stress-induced this interaction and was reduced by TRAF2 and TRAF6 double knockdown [99]. *These findings illustrated that ROS stimulates the dissociation of TRX from ASK1 while assisting the recruitment of TRAF2/TRAF6 to ASK1 signalosome to facilitate the ASK1 autophosphorylation* [92].

The TRAF6 regulates a wide range of signaling pathways in adaptive immunity, innate immunity, bone homeostasis, and antiviral response [99]. It interacts with ASK1 at regions 384 to 655 AA residue [99]. However, the exact interface residues of ASK1 and TRAF6 complexes are also unknown. Therefore, the possible interface residues of TRAF6 responsible for interacting with ASK1 were explored based on the available crystal complexes. The complexes of TRAF6 binding peptide from RANK, CD40, MAVS, and TIFA reveal how it mediates the various signaling cascades [126]. These peptides (from RANK, CD40, MAVS, and TIFA) interact with the TRAF domain (350–499) and share common residues P468 and G472 at its interface (Fig. 6B). Moreover, residue R392 was common among all binding partners such as RANK, MAVS, and TIFA, and another residue, L457 was also common among RANK, CD40, and MAVS (Fig. 6B). On the other hand, UBE2N interacts with the ring domain (50–130) of TRAF6 (Fig. 6B). Consequently, the main *hot spot* residues in TRAF6 are P468, and G472, which are involved in binding and controlling several signaling pathways and may also be involved in ASK1 binding.

6.4.2. Interaction with USP9X

The Ubiquitin-proteasome system is another ASK1 positive regulator under oxidative stress [115]. ASK1 is ubiquitinated and degraded under exposure to oxidative stress. The study has reported that ASK1 binds to Ubiquitin-specific peptidase 9X-linked (USP9X) and led to the deubiquitination of ASK1 in oxidative stress, which further led to the stabilization of ASK1. Additionally, oxidative stress-induced ASK1 activation is repressed in USP9X knockdown cells. Hence, these investigations exemplify the contribution of USP9X in the sustained activity of ASK1 in oxidative stress [115]. It's been reported that USP9X physically interacts at the C-terminal (residues 1295–1374) of ASK1, but the interacting interface residue of both remains elusive. Furthermore, any crystal complexes of USP9x with other proteins are not reported to date.

6.4.3. Interaction with TRIM48-PRMT1

Recently Hirata et al. (2017) reported that tripartite motif 48 (TRIM48) facilitates the ASK1 activation by promoting the K-48 linked polyubiquitination and degradation of its interacting partner protein arginine methyltransferase 1 (PRMT1) [127]. The PRMT1 negatively regulates the activation of ASK1 by supplementing the ASK1-TRX interactions. The TRIM48 knockdown in the mouse xenograft model suppressed the ASK1 activation and cell death by increasing the ASK1-TRX interactions both under normal and under oxidative stress conditions; in contrast, forced expression resulted in ASK1-dependent cancer cell death. Overall, this study suggested that TRIM48 promotes the ubiquitination-dependent degradation of PRMT1, which prevents the ASK1-TRX interactions and thus leads to enhanced activation of ASK1 [127]. Moreover, decreased expression or activity of TRIM48 may upregulate the PRMT1 expression or activity and lead to the development and progression of cancer by inhibiting the apoptosis of cancer cells [127-129]. Therefore, PRMT1 can be an excellent therapeutic candidate to explore the hyperactive state of ASK1. The PRMT1 was also reported to interact with the C-terminal (residues 1217–1374) of ASK1 with unknown exact interacting interface residue between them. In addition, no complex of PRMT1 with other proteins has been reported yet.

6.4.4. Interaction with TNFAIP3

Peng et al. (2017) recognized endogenous controllers of ASK1 movement in hepatocytes during NASH using unbiased mass spectrometry and discovered TNFAIP3 as a vital part of the ASK1 signalosome [43]. In this study, they have reported that polyubiquitination is dependent on ASK1 activation in NASH progression. They found that TNFAIP3 deubiquitination balances the hyperactivation of ASK1 and, along these lines, hinders the improvement of NASH [43]. From *in vitro* and *in vivo* studies, it was reported that TNFAIP3 mediates the suppression of ASK1-p38-JNK1/2 signaling, hence inhibiting the progression of NASH. They also verified the therapeutic potential of TNFAIP3-mediated deubiquitination of ASK1 in NAFLD and NASH models in mouse and cynomolgus monkey (*Macaca fascicularis*) models of NAFLD and NASH [43]. Notably, polyubiquitination is prompted when lipid accumulation outreaches the pathological level but not via physiological stimuli. Along these lines, it was hypothesized that stimulation of the polyubiquitination pathway should largely avoid mitigating and suppressing the normal physiological function of ASK1 and, subsequently, potential undesirable side effects. Through this way of retaining the normal ASK1 activity, TNFAIP3-mediated ASK1 deubiquitination may hold the lead in facilitating proteasome-mediated ASK1 degradation [27] or inhibitor-mediated non-selective blockage of its kinase activity [27,130]. Henceforth, there is a need for a potential lead compound for potentiating the deubiquitination activity of TNFAIP3. These findings uncover a novel mechanism underlying the regulation of ASK1 activity during/at NASH and recommend a promising methodology for the more explicit treatment of hepatic steatosis, inflammation, fibrosis, and insulin resistance. It's been reported that the N-terminal domain (residues 1–277) of ASK1 is responsible for the TNFAIP3 binding, whereas the ASK1 binding region is located in the N-terminal ovarian domain (residues 1–260) of TNFAIP3 [43].

Various cell processes manage their protein turnover through ubiquitination and deubiquitination and, in this way, are reliant on the interaction between Ubiquitin (Ub) and its interacting partners [131]. The regulation of the NF-kB pathway is critically essential for legitimate cellular homeostasis and is subject to dynamic TNFAIP3 to facilitate Ub editing. TNFAIP3 significantly regulates the NF-kB signaling by removing K63-linked ubiquitin and adding the polyubiquitin, which targets the substrate for degradation. Here, we highlight the two unique interfaces of TNFAIP3 for ubiquitin interaction reported in crystals (PDB IDs: 3OJ4 and 5LRX) (Fig. 6C). The region 605–655 of TNFAIP3 is needed for proteasomal degradation of UBE2N and UBE2D3 and TAX1BP1 interaction with UBE2N and TRAF6 deubiquitination, according to a manual assumption based on sequence similarity of UniProt database annotation. Furthermore, the UniProt database's curated annotation revealed that regions 157–159, 190–192, and 224–227 are needed to interact with Ub. In addition, analyzing the structural interface of TNFAIP3 and polyubiquitin-B (UBB) explored a few more residues (S217, L218, Y252, H255) that seem essential for Ub binding (Fig. 6C) [132]. Furthermore, possibly, these residues may also be essential for ASK1 binding.

6.5. Mitogen-activated protein (MAP) kinases (MKK4, MKK7, MKK3, and MKK6)

The ASK1 homodimerization and autophosphorylation, i.e., the hyperactive ASK1, led to the activation of numerous downstream components, essentially using two fundamental pathways. First, the mitogen-activated protein (MAP) kinase 4 (MKK4) and mitogen-activated protein (MAP) kinase 7 (MKK7) are phosphorylated and further activate c-Jun-N-terminal kinase 1 (JNK1) [133]. The JNK1 has numerous downstream effectors, such as c-

Jun, PPARA, IRS1, and c-Fos, that eventually prompt increased insulin resistance, gluconeogenesis, steatosis, inflammation, and decreased lipolysis [134,135]. Second, ASK1 activates mitogen-activated protein kinase (MAP) kinase 3 (MKK3) and mitogen-activated protein kinase (MAP) kinase 6 (MKK6), which energize the p38 pathway. Cell death, cell differentiation, and inflammation have all been mediated by p38 [136]. The liver milieu is primed for hepatic steatosis, inflammation, and cell death after activation of this signal cascade, representing the core of NAFLD pathogenesis. Besides, this disease might be troublesome since the resulting hepatic steatosis, inflammation, and cell death create a self-regenerative oxidative stress environment, which will only drive additional ASK1 activation and hence NASH progression [135].

MKK4 is a stress-activated signaling enzyme that is enzymatically controlled by ligand or substrate binding and post-translational modification [137]. The active MKK4 phosphorylates and activates both JNKs and p38 kinases via dual phosphorylation on Thr (T) and Tyr (Y) residues in the Thr-Pro-Tyr and Thr-Gly-Tyr motif within the activation loop, respectively [137]. The JNK and p38 kinase activation trigger various biological processes, including cell division, proliferation, and apoptosis [137-140]. In advanced prostate cancer and ovarian cancer metastases, MKK4 dysregulation occurs [141,142]. The activity of MKK4 is tightly regulated by the molecular machinery, accompanied by structural changes caused by phosphorylation, to sustain homeostasis in living systems. In a non-phosphorylated state, MKK4 has no activity, whereas certain other kinases, such as ERK2 and Lyn, have mild to moderate activity when they are not phosphorylated. The P38 interaction at allosteric sites of non-phosphorylated MKK4 configures its conformation to an auto-inhibition state. The crystal structure in auto-inhibition state demonstrates that conformational changes lead to the formation of a hydrogen bond between histidine 121 (H121) and phosphothreonine (TPO7) of the P38 peptide (Fig. 7A) [143]. The P38 binding to non-phosphorylated MKK4 induces a conformational modification that blocks the putative binding sites of the substrates, explaining the inhibition mechanism of MKK4 activity [143].

The MKK4 was identified as a significant regulator of liver regeneration and could be an important drug target addressing diseases related to the liver by re-establishing its inherent regenerative capacity [146]. MKK4 came up with beneficial effects on hepatocyte regeneration, fibrosis, robustness, and Fas-mediated apoptosis [146]. The molecular level silencing of MKK4 by means of shRNA led to enhanced signaling via ASK1 and MKK7, which led to excessive phosphorylation of JNK1. Subsequently, ETS transcription factor (ELK1) and activating transcription factor 2 (ATF2) phosphorylation increase, further stimulating and energizing hepatocyte proliferation. Moreover, as MKK7 and JNK1 are involved in the signaling network, therefore, they must be considered major off-targets [146,147].

Because MKK4 plays a crucial role in cell growth, differentiation, and inflammation, this kinase could act as an important drug target for different diseases. Only a few inhibitors have been reported for MKK4 with good potency but without selectivity [146-148]. To overcome this issue, recently a novel selective inhibitor with a high affinity to the on-target MKK4 by chopping out the off-target effects of MKK7, JNK1, BRAFwt, MAP4K5, and ZAK [147]. These inhibitors were optimized from FDA-approved BRAFV600E inhibitor PLX4032 by removing structural features having low nM affinity for MKK4 and outstanding selectivity profiles towards main off-targets MKK7 and JNK1 [147]. Overall, the key modulators and pathway-specific targets need to be explored for the controlled tuning of ASK1 in NASH conditions.

The MKK6 mediates the activation of P38 by making physical interaction with it. We describe two crystal complexes (PDB-IDs: 2Y8O and 5ETF) of MKK6 and P38. In both the crystals, P38 binds
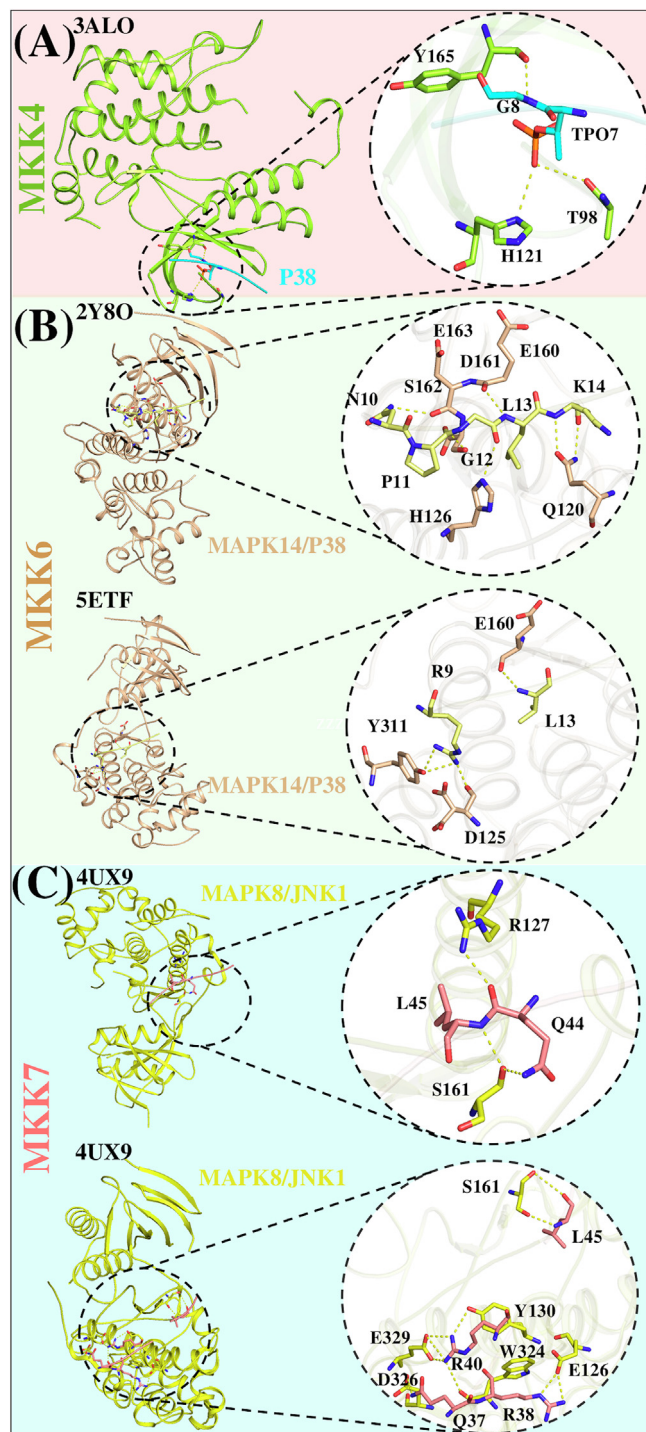
Fig. 7. Interaction analysis of MKK: Schematic representation of (A) MKK4 (lime green color), (B) MKK6 (light brown color), and (C) MKK7 (greenish-yellow color) interaction with their binding partners. The complex's corresponding PDB-IDs are written in black. The protein structure was depicted as a cartoon, while the binding interface residues were shown as a stick format. A zoomed image of the interface residues was also displayed on the right side of the protein structure to provide a clearer vision. Yellow dotted lines depicted hydrogen bond interactions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with the D domain (from residue 4 to 19) of MKK6 protein. The D domain that binds with P38 also binds with ERK2, reflecting that both have a highly similar groove topography. These findings highlighted the limitations of the D domain-mediated interactions. The

activation of p38 by MKK6 likewise relies upon the substrate preference of the MKK6 active site regardless of whether the MKK6 D domain autonomously appears to govern the binding affinity between these two proteins [144]. Moreover, residue Leucine 13 (L13) of P38 was found to be common among both the crystal complexes (2Y8O and 5ETF) of MKK6 and P38 (Fig. 7B). Therefore, it was determined to be a *hot-spot* residue.

Moreover, MKK7 mediates the activation of the JNK pathway, which primarily regulates stress and inflammatory responses. MKK7 makes a more dynamic complex with JNK1 which depends on multi-site interactions to regulate the signaling specificity. Moreover, when MKK7 binds with JNK1, it undergoes conformational exchange from the microsecond to millisecond timescale [145]. This finding corresponds with the crystal structures, in which we noticed two diverse binding sites on the surface of JNK1. In the first binding pose, we observed the hydrogen bond interactions between residue Q44 and L45 of MKK7 and S161 and R127 of JNK1, respectively (Fig. 7C).

Interestingly, at the alternate binding pose, we observed hydrogen bond interactions of positively charged residues R38, Q37, and R40 of MMK7 with residues E126, Y130, W324, D326, and E329 of JNK1, respectively, along with the interaction of L45 of MKK7 and S161 of JNK1 (Fig. 7C). Both observed conformations within the crystal structure suggest that the MKK7 peptide can switch between these two alternate binding sites.

7. Post-translational modifications (PTMs) for ASK1 regulation

The post-translational modifications (PTMs) are well reported to modulate the protein function via dynamically organizing the signaling networks via association/dissociation of PPIs and play a pivotal role in a wide variety of cellular functions [149,150]. The PTMs are known to induce allosteric effects, which are critical for biological function. The PTMs affect the protein function in two ways: (i) orthostatically, through direct recognition by protein domains or interference with binding; and (ii) allosterically, through conformational changes induced at functional sites [151]. Since various chemical forms of PTMs cause different structural changes, the effects of PTM combinatorial codes are much more significant than previously thought [151]. Understanding the functional significance of these modifications in a biological sense includes their recognition, characterization, and mapping to unique amino acid residues on proteins [149]. Many protein modifications, such as ubiquitination, play a critical role in drug response and, ultimately, disease prognosis. As a result, many widely observed PTMs are regularly monitored as disease markers, while others are used as molecular targets in producing target-specific therapies [149]. PTM defects have been related to various developmental disorders and human diseases, emphasizing the role of PTMs in cellular homeostasis [150]. Therefore, among various PTMs, phosphorylation, methylation, ubiquitination, and nitrosylation strongly regulate the activity of ASK1 and, consequently in NASH (Figs. 3 & 8). These PTMs in the context of ASK1 are summarized below.

7.1. Phosphorylation

Phosphorylation of Threonine (T838 and T845) rigidly controls the ASK1 activity, which is mediated by several mechanisms, for example, protein–protein interactions (PPIs) and posttranslational modifications (PTMs) [115]. The ASK1 T838 and T845 phosphorylation occur either by ASK1 *trans*-autophosphorylation or ASK2 in ASK1 signalosome [115]. Additionally, MPK38 has also been reported to phosphorylate T838 in ASK1 and increase the activity of ASK1 [152,153]. MPK38 is also involved in the phosphorylation
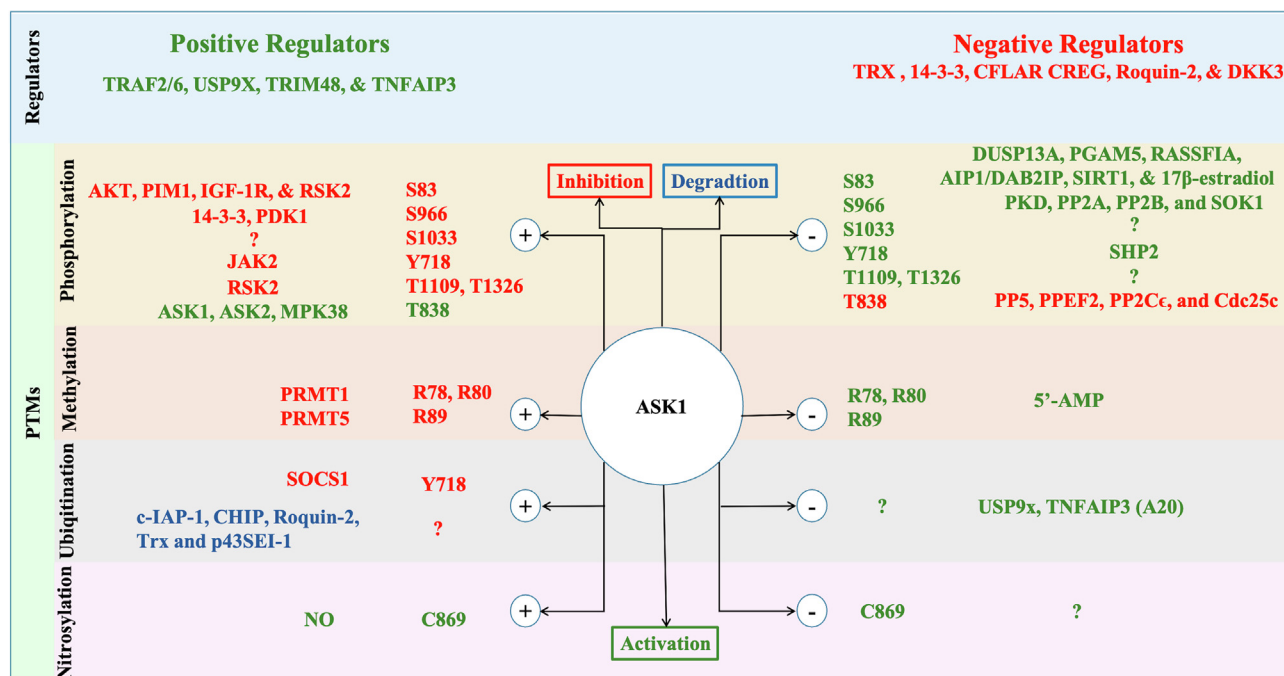
Fig. 8. ASK1 regulators: A schematic diagram of ASK1 regulators (positive, negative, and PTMs).

of serine 188 (S188) of STRAP protein and suppresses its inhibitory effect on ASK1 [154]. Moreover, TRX expression inhibits the MPK38-induced ASK1, P53, and TGF-β. In addition, MPK38 escapes from the TRX-mediated instability by phosphorylating the TRX at threonine 76 (T76) [155], indicating that MPK38 directs the activity of ASK1 through numerous pathways with a positive feedback loop.

Moreover, phosphatases such as PP5, PPEF2, PP2Cε, and Cdc25C negatively controlled the phosphorylation of T838 [156-159]. Under oxidative stress conditions, PP5 interacts with ASK1 and dephosphorylates the phospho-T838 of ASK1 [156]. PP5 activity is regulated by binding partner S100 proteins and the kelch domain containing 10 (KLHDC10) [160]. The S100 proteins enhance the phosphatase activity of PP5 and inhibit the PP5-ASK1 interaction, which in turn leads to ASK1 activation [161]. Under oxidative stress conditions, KLHDC10 interacts with the phosphatase domain of PP5 and impedes its activity hence adding to sustained activation of ASK1 [160]. Hypoxia increases the association of ASK1-PP5 and induces the expression of PP5 by HIF1α activation that inhibits the ASK1 activity [162]. In contrast, the mammalian target of rapamycin (mTOR) inhibition led to the inactivation of PP5 and activation of ASK1 [162,163]. The PPEF2 phosphatase also dephosphorylates the T838 of ASK1 and negatively regulates the activity of ASK1 upon H_2O_2 stimulation [157]. In contrast to PP5, which binds to the activated state of ASK1, PP2Cε binds and maintains ASK1 activity during steady-state conditions [158]. Moreover, Cdc25C dephosphorylates T838 of ASK1 during the interphase of the cell cycle, accordingly inactivating it [159]. However, at the time of mitotic arrest, ASK1 is activated when the binding affinity between Cdc25C and ASK1 diminishes, and the activity of Cdc25C is increased due to hyperphosphorylation. Although further research is required, T838 is not the only phosphorylation site that activates ASK1.

The study has reported that Dyrk1A directly interacts and phosphorylates the ASK1 C-terminal region to activate it [164]. Another study has reported that SLK phosphorylates and positively regulates ASK1, and based on computational prediction analysis, Ser174 in ASK1 is a target phosphorylation site [165]. In addition,

calcium/calmodulin-dependent protein kinase type II (CaMKII) phosphorylates and activates the ASK1 in a Ca2 + -dependent manner [166-170]. In contrast to positive regulation by T838 phosphorylation in the activation loop of ASK1, some phosphorylation sites negatively regulate the activity of ASK1 via possibly altering the conformation and/or their binding pattern. The Akt suppresses the ASK1 activity by phosphorylating the serine 83 (S83) residue of ASK1 [171]. The Hsp90 mediates the interaction between ASK1 and Akt and H_2O_2 treatment changes the composition of the ASK1-Hsp90-Akt complex, in turn, down-regulates S83 phosphorylation and activates the ASK1 [172]. Akt regulates the ASK1 via serine/threonine kinase 11 (STK11), required for the Akt-mediated S83 phosphorylation in ASK1 [173]. DUSP13A, a phosphatase, positively regulates the activity of ASK1 by inhibiting the association between ASK1-Akt [173,174]. A tumor suppressor gene RASSF1A overexpression decreases the S83 phosphorylation and induces expression of ASK1, which results in increased apoptosis induction [175]. Moreover, AIP1/DAB2IP acts as a scaffold protein that regulates cell death and cell survival signaling via positive regulation of ASK1 and negative regulation of Akt [176]. Suppression of ASK1 by Akt has also been reported to be activated by SIRT1 activation [177,178], 17β-Estradiol (E2) signalling [179,180] and ROS signalling [181]. PIM1 is another mediator of S83 phosphorylation, which negatively regulates the ASK1 [181,182]. Another phosphorylation of residue serine 966 (S966) in ASK1 suppresses its activity; in contrast, dephosphorylation of S966 exhibits inverse action and up-regulates the ASK1 activity upon ROS stimulation [85]. The 14-3-3 recognizes and binds to phosphorylated S966 and negatively regulates the ASK1 activity [85,86,183]. Similarly, IKK, a direct kinase, phosphorylates S966 and inhibits signaling of ASK1 upon IGF-1 stimulation, which regulates survival and inflammatory signalling [184]. The IGF-1 suppresses ASK1 activity by phosphorylating S83, S966, and an unidentified Tyrosine (Y) in ASK1 upon IGF-1 signaling [185,186]. Another kinase, PDK1 has also been reported to phosphorylate S966. Moreover, the interaction of ASK1-PDK1 is decreased upon H_2O_2 stimulation of ASK1 [187]. Also, HIV-1 Nef negatively regulates ASK1 by phosphorylating S966 in ASK1 [188]. On the other hand, PKD has been shown to

bind with ASK1 in an H_2O_2-dependent manner and repress the interaction between ASK1 + 14-3-3, which prompts ASK1 activation [172]. Under oxidative stress, Ste20/oxidant stress response kinase 1 (SOK-1) phosphorylates the serine 58 (S58) in 14-3-3ζ, which in turn dissociates 14-3-3ζ from ASK1 and lead to ASK1 activation [189]. In contrast, secreted Klotho increased the phosphorylation of Ser58 in 14-3-3ζ and stabilized the ASK1 + TRX + 14-3-3ζ interaction, prompting the suppression of ASK1 activity [190,191]. PP2A and calcium/calmodulin-activated protein phosphatase calcineurin (PP2B) have been shown to dephosphorylate S966 in ASK1. PP2A is demonstrated to be activated upon TNFα stimulation with AIP1 [192] LPS stimulation [193] and peptidoglycan-induced TLR2 signaling [193,194], while PP2B is demonstrated to be activated in a Ca^{2+}-dependent manner [87,195]. ASK2 is likewise perceived by 14-3-3 using phosphorylation at S964 [196]. The mutant S966A of ASK1 cannot interact with 14-3-3 but doesn't influence 14-3-3 binding with ASK2. However, the S964A mutant or knockdown of ASK2 decreases the ASK1 and 14-3-3 interaction, recommending that ASK2 predominantly modulates the 14-3-3 control of ASK1. Other phosphorylation sites (Y718, S1033, T1109, and T1326) have been reported to regulate the ASK1 negatively. The Tyr718 is phosphorylated by JAK2 and dephosphorylated by SHP2 [196,197]. The SOCS1 recognizes the phosphorylated Y718 and prompts the ubiquitination and degradation of ASK1 using the ubiquitin–proteasome system (He et al., 2006). However, S1033 phosphorylation mechanisms remain unclear; another kinase, RSK2, is reported to phosphorylate S83, T1109, and T1326 and inhibit ASK1 [198]. Phosphorylation of S83 decreases the interaction between ASK1 + MKK6, while phosphorylation of T1109 and T1326 inhibits the interaction between ATP and ASK1 [198]. Moreover, PGAM5, a phosphatase, dephosphorylates the ASK1 inhibitory phosphorylation (s) at sites other than S83, S966, and S1033, and the result activates the ASK1 [70,198]. A study monitored phosphorylation dynamics at twelve potential phosphorylation sites in ASK1 under two distinct stimulations: an electrophilic stress inducer and H_2O_2 and 4-hydroxy-2-nonenal (HNE) [199]. The consequences of this examination demonstrated that the phosphorylation dynamics are distinctive between these two simulations, which proposes that different mechanisms regulate ASK1 phosphorylation among different stimulations.

7.2. Nitrosylation

Nitrosylation regulates the ASK1 activity in similar ways as phosphorylation. Under cerebral ischemia–reperfusion conditions, S-nitrosylation at Cystine (C869) in ASK1 via Nitric oxide (NO) activates the ASK1 [200]. However, at the same site, S-nitrosylation makes ASK1 negative regulation by inhibiting the interaction between ASK1 + MKK3/MKK6 in IFN-γ-treated L929 cells [200,201]. In addition, another study reported that ASK1 S-nitrosylation inhibits its activity under TLR4 signaling conditions, which leads to a negative feedback mechanism [202]. Accordingly, the ASK1 regulatory mechanism by nitrosylation is likely to rely on the interacting proteins under different circumstances [203]. In addition to ASK1 direct nitrosylation, its signaling is regulated by various systems as follows: TRX nitrosylation or nitration inactivates its activity and results in ASK1 activation [204-207], the interaction between ASK1, and Cdc25A (a negative regulator of ASK1) is decreased by nitrosative stress which in turn activates the ASK1 [208], anti-apoptotic protein MCL-1 is degraded by nitric oxide through ASK1-JNK1 signaling and incites cell death [209], reactive nitrogen species (RNS) actuates translocation of TRAF2 and JNK into membrane lipid rafts and prompts to non-apoptotic cell death in an ASK1-, JNK- and TRAF2-dependent manner [210].

7.3. Ubiquitination

Ubiquitination is another core regulatory process that precisely modulates the ASK1 activity. ASK1 degradation by the ubiquitin–proteasome system is an imperative framework that controls ASK1 protein levels. SOCS1, cIAP-1, CHIP, TNFAIP3 (A20), Roquin-2, TRX, and p34SEI-1 have been described to facilitate ASK1 ubiquitination and degradation. The SOCS1 promotes the ubiquitination and degradation of ASK1 by recognizing phosphorylated Tyr718 in the protein [211]. Although ASK1 Tyr718 is phosphorylated in resting endothelial cells, TNFα treatment triggers dephosphorylation at this site, resulting in SOCS1 dissociation and ASK1 activation [211]. It has been described that interferon-gamma (IFN-γ) and interleukin 6 (IL-6) advances the ASK1 ubiquitination and degradation in a SOCS1-dependent manner [197,212]. In contrast, cIAP-1 negatively regulates signaling of ASK1 upon TNFα stimulation in TNFR2 expressing cells [213]. When TNFR2 is stimulated, c-IAP acts as an E3 ubiquitin ligase and ubiquitinates the ASK1, resulting in its degradation. Upon stimulation of TNFα, this framework functions as a negative feedback loop and ends ASK1 signaling [213]. CHIP has additionally been accounted as ASK1 ubiquitin ligase to down-regulate its activity [213,214]. β-arrestin interacts with ASK1 and encourages its ubiquitination in a CHIP-dependent manner [215]. This interaction and ensuing ASK1 ubiquitination are potentiated by H2O2 stimulation and direct the negatively regulated ASK1 signaling. Moreover, after TNFα signaling, CHIP regulates ASK1 negatively [212]. In addition, TNFα stimulation prompts the formation of the HSP70-CHIP-ASK1 complex and advances the ASK1 ubiquitination and degradation. On the other hand, CHIP-dependent ubiquitination of ASK1 is repressed by activated Gα13 [216]. TNFAIP3 also interacts with ASK1 and mediates its ubiquitination and degradation. Upon TNFα signaling and ischemia–reperfusion, TNFAIP3 suppresses the ASK1 signaling [217]. Roquin-2 was recognized as an E3 ubiquitin ligase of ASK1 via a functional siRNA screen [114]. Roquin-2 is needed for ASK1 ubiquitination and controlling its signaling in oxidative stress conditions. Knockdown of Roquin-2 enhances sustained activation of ASK1 and JNK/p38 in response to H_2O_2, which further facilitates cell death. Moreover, TRX, which inhibits the activity of ASK1 by directly interacting with ASK1 as described above, incites ASK1 ubiquitination and degradation through cysteine residue in the active redox site [218]. P34SEI-1 also advances ubiquitination and degradation of ASK1 and suppresses H2O2 -mediated cell death, however a direct physical interaction between these proteins was not noticed [219]. Likewise, ubiquitination mediating the ASK1 negative regulation has also been discovered. USP9X binds with and deubiquitinates ASK1, advancing the ASK1-p38/JNK pathway under oxidative stress conditions [115]. At the C-terminal region, ASK1 contains a ubiquitin-like sequence important for its interaction with USP9X. GSK-3β has been demonstrated to inhibit USP9X, in turn promoting ASK1 degradation that antagonizes ROS-dependent HCC cell death [115,220]. However, upon LPS stimulation Notwithstanding, GSK-3β has additionally been appeared to suppress ASK1 ubiquitination and degradation [221].

Furthermore, there are other types of ubiquitination that regulate the ASK1 activity but do not accompany protein degradation. Fbxo21 advances the Lys29-linked ubiquitination of ASK1 and feasibly activates ASK1 by regulating its phosphorylation at Thr838 [221,222]. The antiviral response requires ubiquitination-dependent ASK1 activation to induce type I interferon production. Covalent modification via ubiquitin-like regulator small ubiquitin-related modifier-1 (SUMO-1) modulates a variety of cellular processes [223], and prevents ASK1 oligomerization that results in suppression of ASK1 [223,224]. The interaction between ASK1 and SUMO-1 is disrupted via H_2O_2 stimulation; however, interestingly, this connection is not a covalent modification.

7.4. Methylation

PRMT1 and PRMT5 have been shown to methylate and negatively regulate the activity of ASK1. PRMT1 does the methylation of arginine (R78) and R80 in ASK1, which inhibits the TRX dissociation and TRAF2 recruitment, leading to the attenuation of ASK1 activity [225]. However, PRMT5 does the methylation of R89 in ASK1 and promotes the interaction between ASK1 and Akt, thus advancing S83 phosphorylation and ASK1 negative regulation [226]. Moreover, in a study, it has been reported that 5'-AMP administration weakens ASK1 methylation and enhances ubiquitination, which therefore prompts ASK1 degradation, inferring that ASK1 methylation could add to its stability [227].

Overall, the knowledge of PTMs can facilitate the pool of other essential interactors which induce and/or remove the PTMs in a protein–protein interaction manner only. The expression, activity, copy number, PTMs, etc., all ways are enriching the pool of other possible interactors of ASK1 in normal and in a pathological condition that can be targeted for therapeutic intervention.

8. Conclusion and future perspectives

1. The increasing prevalence of obesity-related NAFLD and the urgent medical and economic strain of chronic liver diseases such as NASH have challenged modern therapeutics in designing medications to prevent, improve, and reverse liver fibrosis.
2. The promising Gilead's selonsertib, a selective inhibitor of ASK1, which was one of the promising hopes for NASH, has also been shattered as it failed to demonstrate anti-fibrotic efficacy in phase 3 clinical trials. Indeed, Selonsertib is a proven catalytic inhibitor of ASK1; its failure indicated complete inhibition of the catalytic site, possibly not the solution of NASH improvement and/or anti-fibrosis. It led to unraveling the other potential route for NASH. Henceforth, exploring non-conventional routes of drug discovery such as protein–protein interaction interfaces by modulating its endogenous regulators seems to be a potential target.
3. Here, we have extensively explored the regulatory signaling pathways of ASK1 imbalance, as the hyperactive ASK1 is a hallmark of the NASH conditions. ASK1 behavior is controlled by +ve regulators such as (TRAF2/6, USP9X, TRIM48, and TNFAIP3) and −ve regulators (TRX, 14-3-3, CFLAR, CREG, Roquin-2, and DKK3). Also, the PTMs such as phosphorylation, methylation, ubiquitination, and nitrosylation are essential for PPI modulation are broadly discussed.
4. Two ways could modulate the hyperactive ASK1 activity in NASH condition, first, by designing the inhibitory peptides for ASK1 from its −ve regulators directly using the residual information of interacting interfaces of complexes. Secondary, by blocking the positive regulators of ASK1 via inhibiting them through peptides, designed from the interacting interfaces of either ASK1 or from their −ve regulators, which can bind the +ve regulators of ASK1. We postulate the controlled modulation of ASK1 via the PPI approach (i.e., molecular tuning) rather than its total inhibition by modulation of regulators at NASH condition. These findings pave a route towards discovering peptides from various ASK1 regulators of both types, which can occlude either ASK1 interfaces and/or the interfaces of ASK1 regulators at NASH conditions.
5. Among ASK1 regulators, we characterize and deduce all the interactors of 14-3-3 PPI interfaces either in protein or peptide form. It has maximum crystal structure information in terms of regulators, and they share the common key interface residues.
6. From 14 to 3-3 binding partners, a motif RXXpSXP was identified, which can help to generate the common peptides that seem to be a promising approach to tweak the ASK1 behavior. A similar approach can be applied to other regulators as well to identify the potential peptides based on either motif and/or *hot-spot* residues.
7. Similarly, the active *hot-spot* residues of other ASK1 regulators may be used to develop a small molecule using peptidomimetics and based on knowledge of binding grooves the fragment-based approaches. The key amino acids from −ve regulators such as M74 (from TRX) and Q237, E241, E242, N447, L467, Q468, H469, and L471 (from CFLAR), as well as positive regulators like P468 and G472 (of TRAF6), and S217, L218, Y252, H255 (of TNFAIP3), have *hot-spot* residues that could be optimized to produce peptides and peptidomimetic molecules to explore for the reversal of NASH fibrosis.
8. Overall, a PPI-based drug discovery strategy, with the first step being to identify and examine the structural determinants of the protein's interfaces. In the next step, the extracted information will help to design the peptides that are able to bind to the hyperactive state of ASK1 to maintain its usual physiological role.
9. However, the unavailability of complete structural information of ASK1 and experimentally validated knowledge of its regulators remains a significant challenge to explore its critical interface residues that can open an avenue to explore further in order to mitigate the NASH condition and in this regard these curated insights would be beneficial for therapeutic interventions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

S. Asthana conceptualized the study. M. Dikshit provided valuable inputs in understanding the biology of the system. A.K. Agrahari curated the literature and generated the data. A.K. Agrahari and S. Asthana established the rationale. A.K. Agrahari and S. Asthana wrote the manuscript. All authors have read and approved the manuscript.

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