Unravelling molecular mechanisms in transcription-coupled nucleotide excision repair

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Transcription-coupled DNA repair: from mechanism to human disorder

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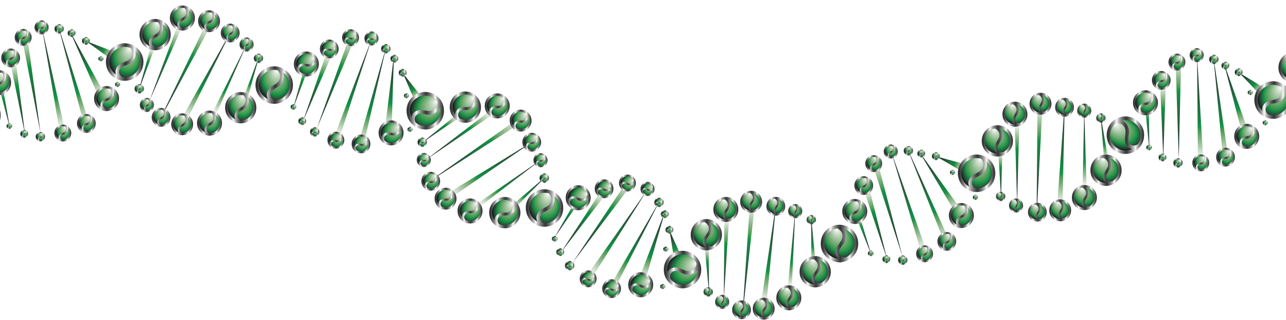

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Abstract

DNA lesions pose a major obstacle during gene transcription by RNA polymerase II (RNAPII) enzymes. The transcription-coupled DNA repair (TCR) pathway eliminates such DNA lesions. Inherited defects in TCR cause severe clinical syndromes, including Cockayne Syndrome (CS). The molecular mechanism of TCR and the molecular origin of CS have long remained enigmatic. Here we explore new advances in our understanding of how TCR complexes assemble through cooperative interactions between repair factors stimulated by RNAPII ubiquitylation. Mounting evidence suggests that RNAPII ubiquitylation activates TCR complex assembly during repair, and in parallel promotes processing and degradation of RNAPII to prevent prolonged stalling. The fate of stalled RNAPII is therefore emerging as a crucial link between TCR and associated human diseases.

The response to DNA damage-induced transcription stalling

The timely and coordinated expression of genes is essential for life. The transcription of protein-coding and non-coding genes involves **RNA polymerase II (RNAPII)** (see Glossary) enzymes, which synthesize RNA transcripts complementary to the DNA template strand. The presence of DNA lesions in the template strand, such as those generated by ultraviolet (UV) light or platinum-based compounds, cause stalling of **elongating RNAPII (RNAPIIo)** (Fig. 1a) leading to a genome-wide transcriptional arrest [2–5]. It is essential that cells overcome this arrest and restore transcription to maintain organized gene expression and viability [5, 6].

The transcription-coupled DNA repair (TCR) pathway efficiently eliminates transcription-blocking DNA lesions from the template stand of active genes in a manner that requires the coordinated association of TCR-specific repair proteins with DNA damage-stalled RNAPII [7–12]. The stalling of RNAPII shields the DNA lesion from direct access by DNA damage-recognition proteins, explaining why TCR is initiated by the recognition of the stalled RNAPII complex, rather than by proteins acting on the DNA lesion itself. While lesion-stalled RNAPII is the initial recruitment platform of TCR proteins, the polymerase ultimately prevents downstream repair proteins to access and remove the actual lesion, suggesting that the processing and removal of RNAPII is intimately linked with the progression of TCR.

Intriguingly, inherited bi-allelic mutations in TCR genes give rise to strikingly different clinical phenotypes, including **Cockayne syndrome (CS)**, which is characterized by severe and progressive neurodegeneration and developmental delay, and **UV-sensitive syndrome (UV^{S}S)**, which is characterized by mild cutaneous photosensitivity without neurological features [4]. Advances in methods to isolate TCR complexes and study their assembly mechanisms [11–13], development of new techniques to directly measure TCR-mediated repair [12, 14], approaches to elucidate molecular structures of TCR intermediates [15, 16], and the availability of cutting edge genome-wide approaches to map global transcriptional response to DNA damage [12, 17–19], have revealed key insights into the least understood human DNA repair pathway and its link with human disease. In this review, we discuss these recent advances, and we propose a unifying model that provides an explanation for the strikingly different clinical features associated with TCR deficiency disorders.

Initiation of TCR: the interplay between CSB, CSA, UVSSA and TFIIH

Although the molecular mechanism of TCR has long been enigmatic, several recent studies have provided the first insights into how TCR proteins associate with DNA damage-stalled RNAPII and initiate DNA repair.

Sequential and cooperative assembly of the TCR complex

TCR is triggered when RNAPIIo is unable to translocate past a DNA lesion and stalls (Fig. 1a). This stalling triggers the stepwise recruitment of TCR proteins, starting with Cockayne syndrome protein B (CSB) [11]. The yeast orthologue of CSB, RAD26, associates with and bends DNA upstream of RNAPII and stimulates its forward translocation past smaller obstacles that would otherwise cause RNAPII backtracking and arrest [16, 20]. However, stalling of RNAPII at DNA lesions that cannot be bypassed may stabilize the CSB-RNAPII interaction and initiate TCR through a mechanism that is still largely elusive.

A recent cryo-EM structure revealed that the ATPase domain (lobe 2) of yeast RAD26 interacts with the RPB2 subunit of RNAPII [16]. Studies on human CSB have shown that a C-terminal region, which is absent in yeast, is needed for a stable interaction between CSB and RNAPIIo [21]. Thus, it remains to be established precisely how CSB recognizes and interacts with lesion-stalled RNAPII. In the absence of transcription-blocking lesions, CSB adopts an autoinhibitory conformation in which the N-terminal region of CSB interacts with and inhibits the ATPase domain, which is antagonized by the C-terminal region [22]. Binding of CSB to lesion-stalled RNAPIIo induces a conformational change in the N-terminus of CSB, exposing residues in the C-terminus, possibly to promote its stable association with RNAPIIo [22].

The exposed C-terminus of CSB also contains a ubiquitin-binding domain (UBD) of unknown function [23], and a recently identified and highly conserved **CSA-interacting motif (CIM)** (Fig.1b, c) [11]. The DNA damage-induced conformational change that exposes the C-terminus of CSB may enable it to interact with and target CSA to lesion-stalled RNAPIIo [11]. The CSA protein functions as the substrate-recognition subunit of a **DDB1-CUL4A-RBX1 ubiquitin ligase complex (CRL4^{CSA})** [24, 25]. While CRL4^{CSA} is inhibited by its association with the COP9 signalosome in undamaged cells, the ubiquitin ligase function of the CRL4^{CSA} complex is activated in response to DNA damage, resulting in CSA auto-ubiquitylation and ubiquitylation of target substrates, including CSB and RNAPII [12, 24–27]. Since the CIM in CSB is located right next to the UBD in CSB (Fig. 1b), it is tempting to speculate that the interaction between CSB and CSA is stabilized by binding of the UBD to auto-ubiquitylated CSA (Fig. 1c). Following its recruitment, CSA promotes the association of the UV-stimulated scaffold protein A (UVSSA) to lesion-stalled RNAPIIo by interacting with the **CSA-interaction region (CIR)** (Fig. 1b, c) located within the N-terminal VHS domain of UVSSA [11, 12, 28]. CSA likely interacts with UVSSA through its C-terminus involving a tryptophan (W361) [29], which is substituted in a patient with a CSA mutation that causes UV^{S}S (Fig. 1b) [30]. It has been suggested that UVSSA may also transiently associate with damaged DNA independently of CSB and CSA shortly after UV irradiation, which is discussed in more detail in box 1.

Box 1. UVSSA association with the TCR complex

The precise manner in which UVSSA is recruited during TCR has been studied using different experimental approaches. A number of studies employed co-immunoprecipitation (Co-IP) of RNAPII typically around one hour after UV irradiation, which revealed that the CSB and CSA proteins are strictly required for the recruitment of UVSSA to DNA damage-stalled RNAPIIo after UV irradiation [11, 12, 28]. Conversely, live-cell imaging experiments visualizing GFP-UVSSA binding within the first 40 seconds after UV-C laser irradiation showed that UVSSA is recruited to sites of UV-C-induced laser damage independently of the CS proteins [10, 32].

There could be several technical reasons for these seemingly conflicting results, including a loss of transiently bound UVSSA during the biochemical isolation of RNAPIIo-associated TCR proteins during a Co-IP, or the recruitment of GFP-tagged UVSSA to sites of UV-C induced laser damages other than stalled RNAPII. Nevertheless, it is possible that UVSSA transiently associates with UV-damaged chromatin independently of the CS proteins as part of a very rapid response, but that the stable association with stalled RNAPIIo during productive TCR is fully dependent on CSA and CSB. Consistent with this explanation, mutants of TCR proteins, such as a UVSSA mutant (ΔCIR) that is unable to associate with CSA [11, 32], is recruited normally to sites of UV-C laser damage [32], but fails to associate with RNAPII in Co-IP experiments [11]. Importantly, this UVSSA mutant fails to support TCR [11, 32]. Although the CS proteins promote the stable association of UVSSA with productive RNAPII-associated TCR complexes, it is certainly possible that the rapid CS-independent recruitment of UVSSA to chromatin has a role that remains to be determined.

The interaction between CSA and UVSSA is stabilized by CSB, suggesting that TCR complex assembly is highly cooperative [11]. Once recruited, UVSSA is the key protein that recruits the **transcription factor IIH (TFIIH)** complex to stalled RNAPIIo [11, 12, 31]. This recruitment occurs through a **TFIIH-interaction region (TIR)** (Fig. 1b, c) partly overlapping the C-terminal DUF domain of UVSSA [11, 29]. Structural analysis revealed that a region within the TIR of UVSSA interacts with the **pleckstrin-homology (PH) domain** of the p62 subunit of TFIIH [31]. Mutating residues within this region (F408A and V411A) impairs the association of TFIIH with damage-stalled RNAPIIo [12], suggesting that UVSSA recruits the TFIIH complex via direct protein-protein contacts with its p62 subunit. Together, these findings reveal a cooperative assembly mechanism of the TCR complex in which the sequential recruitment of CSB, CSA and UVSSA targets TFIIH to stalled RNAPIIo to initiate DNA repair (Fig. 1c).

TFIIH-dependent displacement of RNAPII and completion of DNA repair

The basal transcription factor TFIIH consists of seven core subunits (XPB/p89, XPD/p80, p62, p52, p44, p34, TTDA/p8), and three CDK-activating kinase (CAK) subunits (CDK7, MAT1, cyclin H). In general transcription, TFIIH employs the translocase activity of its XPB subunit and the kinase activity of the CAK complex

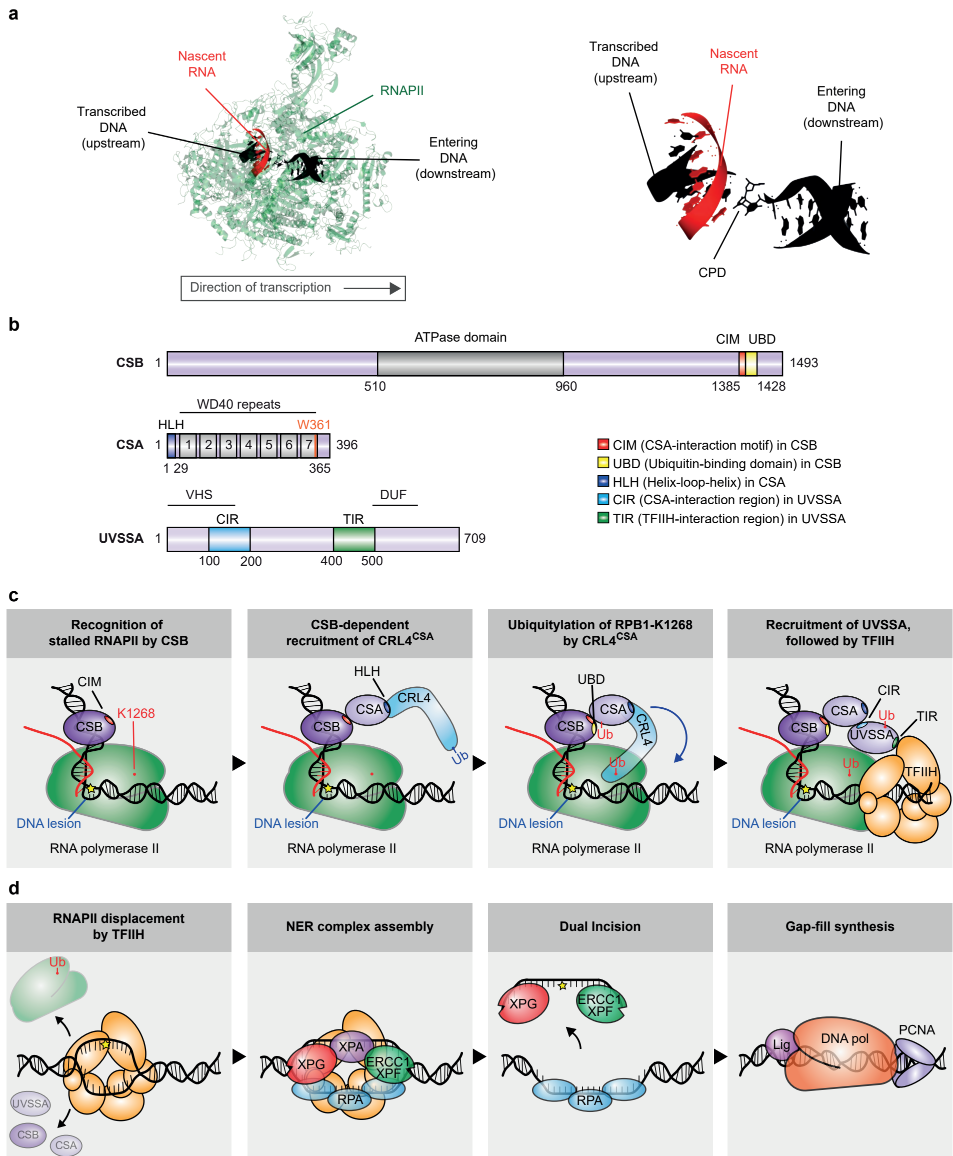

Fig. 1: Molecular mechanism of TCR. (**a**) Cryo-EM structure of RNAPII stalled at a cyclobutane pyrimidine dimer (CPD) lesion (PDB: 2JA5). (**b**) Domain architecture of human TCR proteins, including the domains involved in their interactions. (**c**) Model of TCR complex assembly involving the stepwise and cooperative binding of CSB, CSA, and UVSSA, which together with RPB1-K1268 and UVSSA-K414 ubiquitylation target the TFIIH complex to lesion-stalled RNAPIIo. (**d**) Model of subsequent repair complex assembly involving TFIIH-dependent activities leading to RNAPII displacement, and the subsequent recruitment of XPG, XPA, ERCC1-XPF and RPA, leading to dual incision around the lesion, followed by gap-fill synthesis.

[33]. However, in repair, TFIIH requires the helicase activity of its XPD subunit, which is inhibited by the CAK module [15]. Interestingly, the CAK subunits of TFIIH also associate with lesion-stalled RNAPIIo during TCR [11, 12], suggesting that TFIIH is recruited in this auto-inhibited state. Recruitment of downstream repair proteins XPA and endonuclease XPG by TFIIH subsequently results in dissociation of the CAK module and activation of XPD helicase activity, converting TFIIH from a transcription factor into a repair factor [15, 34, 35].

The combined activity of the XPB and XPD ATPase subunits provide TFIIH with the ability to translocate along DNA and potentially displace DNA-bound proteins. Biochemical evidence using a minimal TCR system suggests that the 3' incision by XPG, which is otherwise blocked by bound RNAPII, requires the ATP-dependent activity of TFIIH to displace RNAPII and gain access to the DNA junction [15, 36]. The need for TFIIH-dependent displacement of RNAPII could also explain why downstream core NER proteins do not associate with stalled RNAPII after UV [11, 16]. Similar to its binding mode in the transcription pre-initiation complex [37], it is likely that TFIIH interacts with the front side of RNAPII during TCR. By utilizing its translocase and helicase activities, TFIIH has been suggested to push RNAPII more upstream of the DNA lesion awaiting repair (Fig. 1c, d). However, more recent genomics approaches suggest that RNAPII dissociates from the DNA template during TCR instead of backtracking [38]. The TFIIH-mediated expansion of the transcription bubble generates torsional stress which could facilitate RNAPII dissociation [39]. Additionally, TFIIH-independent mechanisms could also contribute to RNAPII displacement, including the ubiquitin-dependent extraction of RNAPII from chromatin [6, 17, 40].

Together, these findings suggest that stalled RNAPII serves as an initial platform for the assembly of TCR factors (Fig. 1c), and that TFIIH-dependent and independent mechanisms trigger RNAPII displacement, allowing TFIIH-dependent assembly of a repair complex. Both XPG and XPA stimulate the helicase activity of XPD, suggesting that RNAPII may be rapidly displaced once these two core NER factors are recruited. Subsequent recruitment of ERCC1-XPF by XPA followed by dual incision stimulated by RPA comprise the final steps to remove the DNA lesion before gap filling restores intact DNA (Fig. 1d).

The central role of RNAPII ubiquitylation in regulating TCR

It has been known for quite some time that the largest subunit of RNAPII, **RPB1**, is ubiquitylated in response to UV irradiation in human cells [41, 42], but the precise role of this modification remained enigmatic. Several groups recently identified a single DNA damage-induced ubiquitylation site in RNAPII (RPB1-K1268) [12, 17] with multiple intertwined functions, which can even act sequentially as a molecular timer during repair. Emerging evidence suggests that RNAPII ubiquitylation has a key role in TCR complex assembly, and in regulating a UV-induced transcriptional shutdown. Additionally, the DNA damage-induced ubiquitylation of RNAPII also regulates its processing and degradation, which becomes particularly important under conditions when repair fails, and persistent stalling of RNAPIIo would cause severe toxicity.

Box 2. Ubiquitin ligases involved in RNAPII ubiquitylation

Multiple pathways have been linked to RNAPII ubiquitylation in human cells, including CSA/CSB [12, 27, 41, 42], UVSSA, likely due to its association with deubiquitylase USP7 [9, 10, 28], BRCA1/BARD1 [43], the CUL5-based Elongin A complex [44], and the HECT E3 ligase NEDD4 [45]. RNAPII ubiquitylation is dramatically reduced in CSB-KO and CSA-KO cells at early time-points (1 h) after UV [12, 27], demonstrating that the CSB-dependent recruitment of the $CRL4^{CSA}$ complex to DNA damage-stalled RNAPII [11] is the major contributor to RPB1 ubiquitylation. However, CSA-KO cells still have residual RNAPII ubiquitylation [12], which is fully inhibited by NEDD8 inhibitor [12, 17], suggesting that another CRL-based E3 ubiquitin ligase may also contribute in addition to $CRL4^{CSA}$. A previous study suggested that the loss of RNAPII ubiquitination in CSB/CSA-deficient cells is an indirect effect caused by the general loss of transcription in these cells around 1 hour after UV irradiation [45]. Several experimental findings argue against this explanation. Firstly, recovery of RNA synthesis (RRS) experiments show that both CS-deficient and WT cells shut down transcription to a similar extent at these early time-points after UV [13]. Secondly, XPA-deficient and XPG-deficient cells show normal RNAPII ubiquitylation at 1 h after UV despite their TCR deficiency, demonstrating that the shut-down of transcription in TCR-deficient cells does not cause a loss of RNAPII ubiquitylation [42]. An interesting possibility is that residual RNAPII ubiquitylation in CSA-KO cells is carried out by the sequential activity of the E3 ligase NEDD4 and the CUL5-Elongin A complex [46]. NEDD4 would in this scenario carry out mono-ubiquitylation of RNAPII, while the CUL5-based complex would then be responsible for RNAPII poly-ubiquitylation [46]. This is consistent with the finding that RNAPII poly-ubiquitylation is fully inhibited by NEDD8 inhibitor [12, 17], which inhibits the activity of CRL-based E3 ligases, without affecting HECT E3 ligases, such as NEDD4. Whether the $CRL4^{CSA}$-mediated poly-ubiquitylation of RNAPII is preceded by the mono-ubiquitylation by another E3 ligase remains to be established. Evidence for this scenario comes from findings using di-Gly-Gly proteomics, which cannot distinguish between mono- and poly-ubiquitylation, showing that CSA-KO cells show comparable RNAPII ubiquitylation only at 45 min after UV with strongly reduced levels at later time-points [17]. In contrast, western blot approaches show that RNAPII poly-ubiquitylation is dramatically reduced in CSA-KO cells at 1 h after UV [12].

Ubiquitylation of RNAPII and UVSSA controls TCR complex assembly

Cells impaired in RPB1-K1268 ubiquitylation are unable to restart transcription after UV irradiation, and are strongly impaired in the removal of DNA lesions from transcribed strand as shown by a new strand-specific ChIP-seq method (**TCR-seq**) [12]. These findings demonstrate a direct role of RNAPII ubiquitylation in TCR. Both CSB-KO and CSA-KO cells show dramatically reduced RNAPII ubiquitylation [12, 41], suggesting that the CSB-dependent recruitment of the

CRL4^{CSA} complex to DNA damage-stalled RNAPII [11] is a major contributor to RPB1 ubiquitylation (Fig. 1c, box 2) [12, 27]. It is possible that a CRL-based E3 ligase other than CRL4^{CSA} also has a minor contribution, considering that CSA-KO cells still have a limited amount of residual RPB1 ubiquitylation, which is fully suppressed by NEDD8 inhibitor [12, 17]. The ubiquitylation of RPB1-K1268 is not required for the association of CSB and CSA with lesion-stalled RNAPIIo, but instead stimulates both UVSSA and TFIIH recruitment (Fig. 1c) [12]. This is in agreement with earlier work showing that UVSSA preferentially interacts with ubiquitylated RNAPII, possibly through the VHS domain of UVSSA, which has been implicated in ubiquitin binding [9]. UVSSA is also ubiquitylated in response to DNA damage [10, 47], and recruitment of the K414-ubiquitylated form of UVSSA to lesion-stalled RNAPIIo is particularly dependent on RNAPII ubiquitylation [12]. Precisely how these modifications regulate each other remains to be elucidated. While the protein-protein interaction between UVSSA and TFIIH is not dependent on UVSSA ubiquitylation, UVSSA ubiquitylation appears to stimulate the displacement of p62 from UVSSA, likely to allow TFIIH to be transferred from UVSSA to lesion-stalled RNAPIIo (Fig. 1c) [12]. Altogether, these recent advances emphasize how the stepwise and cooperative assembly of the TCR complex, containing CSB, CSA, and UVSSA, together with RPB1-K1268 and UVSSA-K414 ubiquitylation targets the TFIIH complex to lesion-stalled RNAPIIo to initiate the repair of transcription-blocking DNA lesions (Fig. 1).

RNAPII ubiquitylation and ATF3 regulate a global transcriptional shutdown

The transcriptional response to UV is multifaceted and tightly controlled at multiple levels. Initially, there is a rapid and global shutdown of transcription (1-3 h), followed by the slow recovery of transcription (<24 h) [3]. The shutdown of transcription involves two stages: restriction of elongation to the first 20 kb of genes due to the presence of transcription-blocking DNA lesions (<1 h; Fig. 2a, b) [3, 18], followed by the loss of transcription initiation from transcription start sites (TSS) (<3 h; Fig. 2c). Two key mechanisms have been proposed that regulate the genome-wide loss of transcription initiation from TSS after UV (Fig. 2c) [17, 48, 49].

The transcriptional repressor ***ATF3*** is one of the immediate early response genes whose expression is induced by UV light. Upon its induction, ATF3 associates with CRE/ATF sites, which are located close to promoters of a large number of genes. The ATF3-mediated repression of such target genes was suggested to cause the transcriptional shutdown after UV [50]. This inhibitory impact is relieved by ATF3 ubiquitylation and subsequently degraded by the proteasome in a CSA/CSB-dependent manner, thereby restoring transcription in WT cells at late time-points (24 h) after UV (Fig. 2d) [19]. However, the failure to degrade ATF3 in CS-deficient cells was suggested to underlie their persistent UV-induced transcriptional shutdown, including shutdown of important neuronal genes, potentially explaining the neurodegenerative features described in CS (Fig. 2d) [19, 50, 51]. Indeed, expression of an ATF3 mutant that could not be ubiquitylated led to persistent transcriptional repression of target genes after UV [50]. A testable prediction of this model would be that cells from UV^{S}S patients, who do not suffer from neurodegeneration, would not repress ATF3 target genes after UV irradiation.

Recently, RNAPII ubiquitylation and subsequent degradation was identified as another mechanism to limit transcription initiation after UV (Fig. 2c) [17]. Specifically, initiation shutdown of short genes does not occur efficiently in RPB1-K1268R cells that are impaired in RPB1 ubiquitylation, resulting in persistent expression of short genes, including *FOS* and *ATF3*, after UV [17]. *In silico* modeling suggested that the loss of transcription initiation is the result of proteolytic degradation of RNAPII after encountering a DNA lesions, suggesting that UV-induced depletion of the RNAPII pool below a certain threshold causes a shutdown of transcription initiation [17]. Although intriguing, it cannot be excluded that RNAPII ubiquitylation regulates transcription initiation through other mechanisms that do not require its degradation, especially considering that strong depletion of the entire RNAPII pool is not observed in many studies [9–12, 49]. Nonetheless, subtle changes in the RNAPII pool may already have a significant impact, particularly if specific forms of RNAPII are affected. For instance, elongating RNAPIIo is dephosphorylated to **RNAPIIa**, which can subsequently be recycled to initiate transcription from promoters. Although the overall levels of RNAPII are unaltered in CSB-deficient cells after UV, there is a specific loss of RNAPIIa [49], which could also cause a shutdown of transcription initiation. Even in the absence of exogenous DNA damage, CSB deficiency has been linked to aberrant regulation of a number of neuronal genes [52]. It would be interesting to see whether RPB1-K1268 ubiquitylation affects the dephosphorylation of RNAPIIo allowing the recycling of RNAPII during the transcription cycle.

The proposed mechanisms for the UV-induced transcriptional shutdown involving ATF3 [19, 50, 51] and RNAPII ubiquitylation [12, 17] show many similarities. One interesting possibility is that ATF3 and RPB1-K1268 ubiquitylation operate in a common pathway. Knock-out of ATF3 causes a failure to recruit CSB, the $CRL4^{CSA}$ complex, and the proteasome to the CRE/ATF site close to the promoter of several genes, including neuronal genes [19, 50, 51]. This is accompanied by a failure to remove RNAPII at these promoter regions, which does occur normally in WT cells after UV [19, 50, 51]. Based on this, it is possible that ATF3 could recruit the $CRL4^{CSA}$ complex not only to degrade itself, but also to promote RNAPII ubiquitylation around promoters, leading to a shutdown of transcription initiation through RNAPII degradation, or another mechanism (Fig. 2c).

Interestingly, ectopic expression of RPB1-K1268R or knock-down of ATF3 in CSB-deficient cells restored expression of a number of genes after UV [17, 19, 50]. This raises the question how transcription can be restored, despite the inability of CSB-deficient cells to eliminate transcription-blocking DNA lesions from these genes. It will be important to directly measure repair activity in these genes using TCR-seq [12], to determine if DNA lesions are eliminated through an alternative mechanism despite the absence of CSB under these conditions. Understanding the UV-induced transcriptional shutdown and the precise interplay between ATF3 and RNAPII ubiquitylation in this process will require the development of more sensitive methods and specific antibodies to detect RPB1-K1268 ubiquitylation.

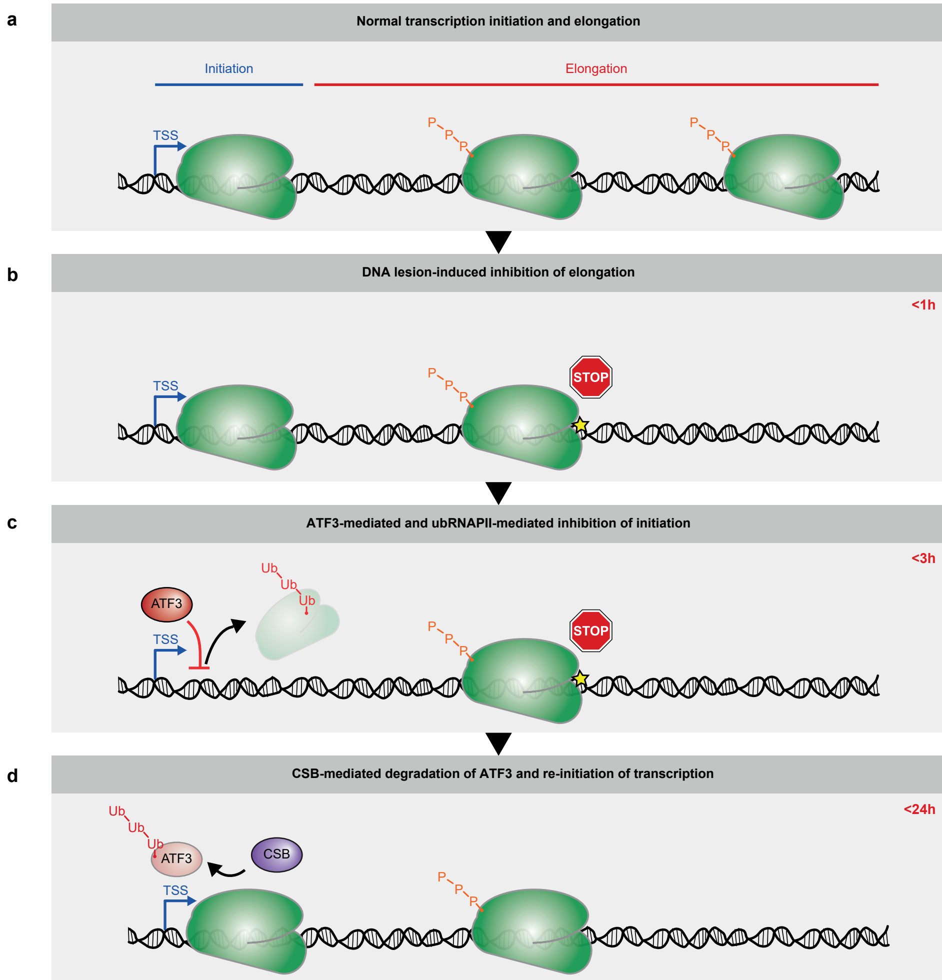

Fig. 2: Mechanisms Involved in the UV-Induced Shutdown and Restart of Transcription. (**a**) The transcription cycle involves transcription initiation at TSS followed by transcription elongation. (**b**) Shortly after UV (<1 h), transcription elongation becomes restricted to the first 20 kb of genes due to the presence of transcription-blocking DNA lesions, which prevent RNAPII from progressing further into genes. (**c**) This inhibition of transcription elongation (**b**) is followed (<3 h) by a loss of transcription initiation from TSS due to ATF3-mediated repression and RNAPII ubiquitylation, which may be part of a common molecular pathway. (**d**) Following successful repair, cells recover transcription (<24 h), which involves the CSB/CSA-dependent removal of ATF3 from TSS and likely other unknown mechanisms.

Ubiquitylation of RNAPII allows removal of persistently stalled RNAPII

Multiple ubiquitin ligases have been linked to RNAPII ubiquitylation after UV (box 2). Recently, a number of studies have shown that CRL4^{CSA} is a key ubiquitin ligase that contributes to RNAPII ubiquitylation after UV [12, 27]. This is in line with earlier work showing that RNAPIIo ubiquitylation and degradation do not occur in cells deficient in CSA or CSB [41], while RNAPIIo ubiquitylation is normal in cells deficient in more downstream TCR genes, such as XPA, XPG, and XPF [42].

The CRL4^{CSA} complex readily associates with RNAPIIo after UV irradiation in a manner that depends on CSB [11], providing an explanation for the requirement of both CS proteins in this process (Fig. 1c, Fig. 3a). A previous study suggested that the loss of RNAPIIo ubiquitylation in CSB/CSA-deficient cells is an indirect effect caused by a general loss of transcription shortly after UV [45], but this idea is not supported by recent data showing that RNAPIIo levels are not considerably reduced in CSA/CSB-deficient cells shortly after UV. A more likely explanation is that cells deficient in CSB and CSA are unable to degrade RNAPII after UV, because RNAPII is not poly-ubiquitylated to begin with (box 2) [9, 11, 12, 41, 42]. UVSSA-deficient cells show faster RNAPII degradation after UV [9], suggesting that the recruitment of UVSSA and its interactor **USP7** mediate the de-ubiquitylation of RNAPII to prevent its proteasomal degradation while TCR is ongoing (Fig. 3b) [9, 28]. Thus, RNAPII ubiquitylation by CSB and CRL4^{CSA} acts as a molecular timer during repair. The recruitment of UVSSA-USP7 results in trimming of the ubiquitin chains deposited by CRL4^{CSA} to prevent untimely degradation of RNAPII. In UVSSA-deficient cells, RNAPII processing is still possible, because RNAPII has been marked by ubiquitylation (Fig. 3b). However, the inability to ubiquitylate RNAPII in cells defective in CSB and CSA will lead to persistent stalling of RNAPII (Fig. 3c). These striking differences in RNAPII processing affect clinical phenotypes associated with TCR deficiency, which is discussed in the next section.

The origin of Cockayne syndrome

Inherited defects that selectively impair TCR give rise to CS and UV^{S}S. The majority of CS patients carry mutations in the *CSB* or *CSA* genes [53, 54], while UV^{S}S patients carry mutations in the *UVSSA* gene [9, 28]. Rare cases of UV^{S}S with mutations in the *CSB* [55] or *CSA* [30] genes have been reported. The clinical phenotypes associated with these TCR disorders are very different. CS is characterized by developmental abnormalities combined with severe and progressive neurodegeneration, caused by demyelination, and progressive loss of neurons [56, 57]. In contrast, UV^{S}S is characterized by mild UV sensitivity without devastating neurodegenerative features seen in CS [30, 55, 58]. Several disease mechanisms have been proposed to contribute to progeroid features in CS, including defects associated with transcription misregulation [17, 48, 51, 52], neuronal development [51, 59], repair of oxidative damage [60], and mitochondrial function [61–63]. As discussed above, recent insights into the molecular mechanisms of TCR reveal that CSB, CSA and UVSSA are all essential for TCR, and that UVSSA acts downstream from CSB and CSA, to recruit the TFIIH complex to DNA damages-stalled RNAPII (Fig. 1c) [11, 12]. These findings demonstrate that defective removal of transcription-blocking DNA lesions by TCR does not explain the severe clinical phenotype associated with CS.

Rather than a DNA repair disorder, we propose that CS is caused by a deficiency in RNAPII processing. Improper RNAPII ubiquitylation might impair removal of RNAPII at promoter regions of numerous genes, which combined with persistent ATF3-mediated transcriptional repression in the absence of CSA or CSB, cause aberrant gene expression [50–52]. Moreover, impaired RNAPII ubiquitylation results in its prolonged arrest at DNA lesions. Together, these

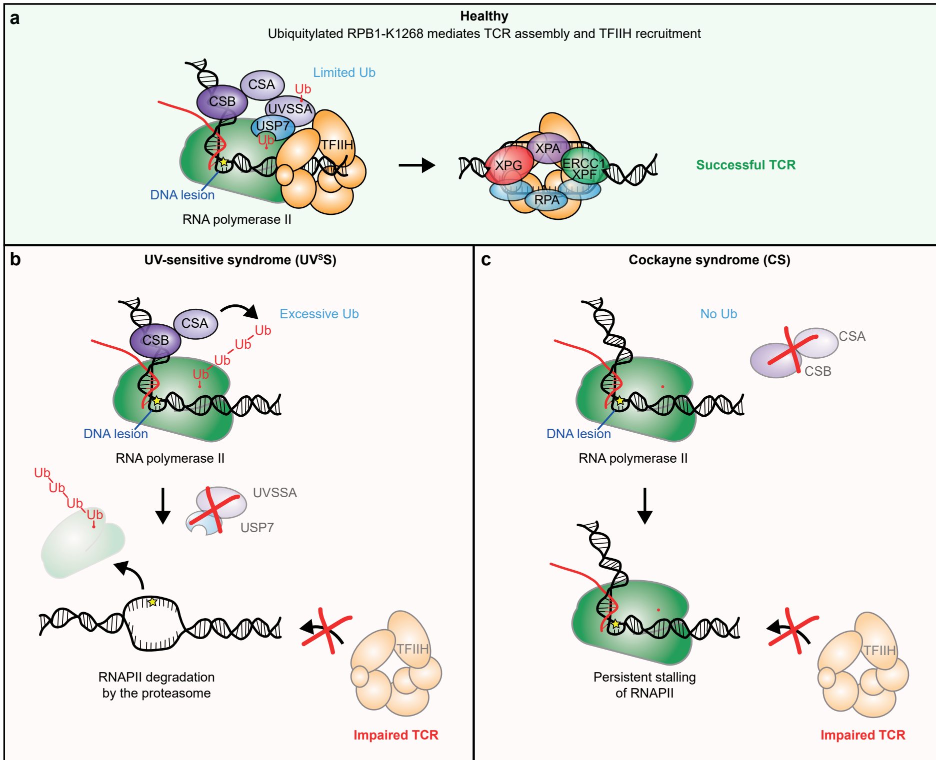

Fig. 3: Differential processing of RNAPII during normal and defective repair explains clinical features associated with TCR deficiency disorders. (**a**) CS protein-dependent ubiquitylation of RPB1-K1268 acts as a molecular clock that promotes DNA repair at early time-points. The recruitment of UVSSA-USP7 during repair limits RNAPII ubiquitylation. The UVSSA-dependent recruitment of TFIIH, and the ensuing activation of TFIIH, results in displacement of RNAPII and enables successful repair. (**b**) In the absence of UVSSA, both CSB and $CRL4^{CSA}$ are recruited normally to stalled RNAPIIo, resulting in RNAPII ubiquitylation. Without trimming by UVSSA-USP7, excessive RNAPII ubiquitylation leads to accelerated processing and degradation of RNAPII. Though impaired in TCR, this prevents toxicity associated with persistent RNAPII stalling, resulting in $UV^{S}S$. (**c**) Inactivation of CSB or CSA function leads to a failure to ubiquitylate and subsequently degrade DNA damage-stalled RNAPIIo. Persistent and prolonged stalling of RNAPIIo not only impairs TCR, but also blocks the accessibility of DNA lesions to alternative repair pathways, and causes profound toxicity and neurodegeneration seen in CS.

effects might ultimately lead to the clinical manifestations of CS, including neurodegeneration. In this model, the CS protein-dependent ubiquitylation of RPB1-K1268 acts as a molecular clock that promotes DNA repair and shutdown of transcription at early time-points (Fig. 1c, Fig. 3a), but when repair fails it will lead to processing and removal of DNA damage-stalled RNAPII (Fig. 3b, c). In line with this model, CSA or CSB-deficient primary fibroblasts fail to ubiquitylate and subsequently degrade RNAPIIo [9, 41], while UVSS fibroblasts displayed even faster degradation of RNAPIIo after UV, possibly due to a failure to deubiquitylate RNAPIIo by the UVSSA binding partner USP7 (Fig. 3b) [9, 10, 28, 29]. A recently established RPB1-K1268R knock-in mouse deficient in the DNA damage-induced ubiquitylation of RNAPII indeed shows striking features of CS, including

pronounced dwarfism, growth retardation, severe neurodegeneration and short life-span [12]. These findings suggest that clinical features of CS are caused by toxicity associated with prolonged RNAPIIo stalling at lesions, which blocks accessibility of the DNA lesions to alternative repair pathways. This unifying model may provide a long sought-after explanation for the strikingly different clinical features associated with TCR deficiency disorders (Fig. 3a-c).

A recent study showed that ectopically expressed RPB1 is rapidly degraded in CSB-deficient cells after UV exposure (3 h), which did not occur when RPB1-K1268 was mutated [17]. Based on these findings, it was suggested that rapid degradation of the entire RNAPII pool, resulting in aberrant gene expression, underlies clinical features of CS [17]. Conceptually, however, this model is unlikely as it would not explain (I) why UVSSA-deficiency, which is associated with fast degradation of RNAPII after UV [9, 28], does not cause CS-like features, and (II) why the RPB1-K1268R knock-mouse, which is unable to degrade RNAPII, shows a CS-like phenotype. Most importantly, although this phenotype was observed for ectopically expressed RPB1 [17], several other studies found that the degradation of endogenous RPB1 does not occur in CSA or CSB-deficient cells [9, 41, 45].

Although many studies in CSA or CSB-deficient fibroblasts have focused on responses to UV light, it is currently unclear which endogenous DNA lesions cause CS-like features. We speculate that aldehydes and oxygen-induced cyclopurines are likely endogenous DNA lesions in the brain that block RNAPII progression, and may strongly contribute to a CS-like phenotype due to impaired RNAPII processing. Similarly, defects in processing of RNAPII at various types of DNA damage may contribute to the development of neurodegenerative phenotypes shared among genome-instability disorders [64].

Concluding remarks

For decades, the lack of sensitive biochemical, proteomics or genomics methods has hampered our scientific progress in unraveling mechanisms in transcription-coupled DNA repair. Over the past few years, numerous new techniques have been developed that enabled us to reveal long sought-after insights into the mechanisms underlying TCR. This review focused on recent insights ranging from the initial DNA damage-induced transcription shutdown, the sequential and cooperative assembly of TCR complexes that remove transcription-blocking DNA lesions, followed by the recovery of transcription after repair has been completed. We focused specifically on the role of DNA damage-induced ubiquitylation of RNAPII in each of these steps, and propose a unifying model explaining the strikingly different clinical features associated with TCR-deficiency disorders. While involved in shutting down transcription and activating the TCR complex assembly during early DNA repair, RNAPII processing and degradation might also be crucial when repair fails, ultimately defining the fate of RNAPII and the clinical features seen in TCR-deficiency disorders, such as Cockayne syndrome.

Although these recent insights provide a good starting point, major efforts will be needed in the coming years to further unravel the complexity of TCR and its interconnection with DNA damage-induced transcriptional responses. Ultimately, integrating this knowledge with emerging functional roles of CS proteins in other

processes [48, 52, 61–63] will provide a better understanding of the molecular origin of Cockayne syndrome. Several key questions remain unanswered and will be the major focus in the years to come (see Outstanding Questions). Powerful methods including proximity-dependent biotin identification (Bio-ID) [65], genome-wide CRISPR screens [66], and proteomic approaches [32, 67] will not only identify the full repertoire of components required for TCR, but will finally enable *in vitro* reconstitution and ultimately structural analysis of the various compositions of the TCR complex by cross-linking mass spectrometry (XL-MS) [68] and cryo-electron microscopy (cryo-EM) [15]. The development of site-specific proteomics approaches will be needed to define the order of events during TCR at individual lesions. As it is becoming increasingly clear that successful DNA repair alone is not sufficient to restore transcription, we need a better grasp of the mechanisms and the choreography of DNA damage-induced transcriptional responses. With roles for ATF3-mediated transcriptional repression [19, 50, 51], changes in RNAPII phosphorylation status affecting RNAPII elongation rates [49], and ubiquitylation-mediated release or degradation of RNAPII [12, 17], these processes now start becoming more and more into focus. The next few years will certainly bring exciting answers to these fundamental questions enabling us to bring the molecular mechanism of transcription-coupled DNA repair and its link with human disorders to light.

Box 3. Additional regulators of the UV-induced transcriptional response

Changes in phosphorylation of the carboxy terminal domain (CTD) of RPB1 have been linked to global downregulation of transcription [48, 49, 69, 70]. A recent study demonstrated that UV-induced hyperphosphorylation of RNAPII by glycogen synthase kinase 3 (GSK-3) leads to reduced RNAPII elongation rates, which affect alternative splicing [69], probably due to increased time to use suboptimal splice sites [70]. Treatment with GSK-3 inhibitors prevented UV-induced changes in transcription elongation rates and alternative splicing, and abolished RNAPII hyperphosphorylation without affecting general RNAPII phosphorylation, demonstrating that GSK-3 specifically phosphorylates RNAPII in response to UV irradiation [69]. Interestingly, phosphomimetic mutations on either Ser2 or Ser5 that mimic RNAPII hyperphosphorylation result in slower RNAPII elongation and affect alternative splicing similarly to UV irradiation, which confirms that disrupted phosphorylation patterns are involved in the global downregulation of transcription [70]. In addition, several other regulatory factors are implicated in TCR, UV-induced transcriptional shutdown and/or transcription restart, including UBR5 and the Polycomb repressive complex (PRC1) [71], SPT16 [32], SNF2H [72], HIRA [73], ELL [74], and DOT1L [75], but their precise mode of action or direct role in repair remains to be elucidated. Although the nucleosome-binding protein HMGN1 has often been implicated, recent studies show that human HMGN1 and HMGN2 have no role in TCR [13]. Finally, recent siRNA screens [67], and genome-wide CRISPR screens [66] identified STK19 and ELOF1 as potential TCR factors, although their roles are currently elusive.

Outstanding Questions

Although major steps have been taken in unraveling mechanisms in TCR and its interconnection with DNA damage-induced transcriptional responses, several questions remain:

- How does CSB recognize DNA lesion-stalled RNAPII and discriminate it from paused RNAPII?
- How does repair progress once RNAPII has been released? Does backtracking in front of the lesion occur, or is release of RNAPII universal?
- Which additional E3 ubiquitin ligases, in addition to $CRL4^{CSA}$, mediate RPB1-K1268 ubiquitylation, and how do they cooperate?
- What is the role of UVSSA ubiquitylation and how does RNAPII ubiquitylation meditate the hand-over of TFIIH onto DNA lesion-stalled RNAPII?
- Do we know all components required for TCR (box 3)? This is unlikely considering that recent genetic screens uncovered numerous new proteins, including SPT16, HIRA, DOT1L, STK19 and ELOF1 [32, 66, 67, 73, 75]. What is their role, and do they represent new core TCR components?
- How do cells restore transcriptional activity once DNA repair has been completed? Which signaling pathways regulate this?
- How is the expression of a subset of genes after UV-induced DNA damage restored in CSB-deficient cells when ATF3 is depleted, or an RPB1 version that cannot be ubiquitylated is expressed? Can TCR operate without CSB under these conditions? Does an alternative pathway eliminate transcription-blocking DNA lesions under these conditions?
- Are all clinical features of CS independent of TCR?

Glossary

Activating transcription factor 3 (ATF3): A stress-induced transcriptional repressor that binds specific DNA sequences (CRE/ATF sites) and thereby regulates the expression of its target genes.

Cockayne syndrome (CS): A severe human disorder characterized by progressive neurodegeneration and developmental abnormalities mostly caused by mutations in the genes encoding *CSB* and *CSA*.

Cullin-RING E3 ubiquitin ligase (CRL4): E3 ligase complex composed of CUL4A/B, DDB1, and RBX1, which binds specific adapters, such as CSA, to confer specificity.

CSA-interacting motif (CIM): An evolutionary conserved motif in CSB (amino acids 1385-1399) that is required for its association with CSA.

CSA-interacting region (CIR): A region in UVSSA (amino acids 100-200) that is required for its association with CSA.

Pleckstrin-homology (PH) domain: A motif of ~120 amino acids that is present in various signaling proteins, which can interact with other PH-binding proteins that contains a track of acidic residues, such as UVSSA.

RNA polymerase II (RNAPII): A multi-protein complex made up of 12 subunits (~550 kDa) that transcribes DNA into a complementary RNA molecule. The largest subunit of RNAPII, **RPB1**, contains a C-terminal domain (CTD) that is phosphorylated during the transcription cycle; while Ser5 is phosphorylated during transcription initiation, Ser2 is phosphorylated during the transition from transcription initiation to transcription elongation, followed by a progressive loss of Ser5 phosphorylation towards the end of genes. RPB1 is predominantly ubiquitylated at a single lysine residue (RPB1-K1268) in response to UV irradiation.

TCR-seq: A strand-specific RNAPII ChIP-seq method that enables a genome-wide quantification of TCR kinetics.

Transcription factor II H (TFIIH): General transcription factor and key repair complex that consists of 10 subunits. The 7-subunit core complex (containing the XPD and XPB helicases) is sufficient for repair, while the 3-subunit CDK-activating kinase (CAK) complex (containing the CDK7 kinase) is also essential during transcription initiation. The CAK complex dissociates from core TFIIH during repair.

TFIIH-interacting region (TIR): A region in UVSSA (amino acids 400-500) that is required for its association with TFIIH.

UV-sensitive syndrome ($UV^{S}S$): Mild disorder characterized by UV sensitivity without neurological symptoms, predominantly caused by mutations in the gene encoding *UVSSA*.

Ubiquitin-specific protease 7 (USP7): Deubiquitylating enzyme with a wide range of substrates that associates with UVSSA and has a role during TCR, such as stabilizing CSB.

Vps27-Hrs-STAM (VHS) domain: Domain found in the N-terminus of UVSSA.

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