UNIVERSITY OF CRETE- DEPARTMENT OF BIOLOGY, SCHOOL OF MEDICINE

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Delineating the role of XAB2 in mammalian physiology

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Contents

Abstract

To maintain genome stability, which is continuously challenged by a variety of environmental and endogenous agents, cells have evolved a battery of DNA damage signaling and repair machineries. Nucleotide excision repair (NER), is a highly conserved pathway which repairs a broad class of helix-distorting lesions. NER defects are associated with cancer and premature aging, yet the neurological, developmental and metabolic abnormalities observed in NER syndromes cannot be explained only by the repair deficiency, which implies that NER factors function also beyond repair. Indeed, it is currently known that NER factors are also involved in transcription, chromatin remodelling and possibly pre-mRNA splicing. The possibility of a functional interplay between DNA damage repair/response and pre-mRNA splicing has recently emerged. Pre-mRNA splicing factors have been shown to either regulate, at the post-transcriptional level, the proper expression of DNA Damage Response (DDR) genes or be directly recruited at the site of damage. A protein linking pre-mRNA splicing with DNA repair is XPA Binding protein 2 (XAB2), which has been involved in NER, transcription and pre-mRNA splicing, its exact function, however, remains elusive. In order to investigate the functional contribution of XAB2 in hepatic physiology, biotin-tagged XAB2 mice were generated to allow the in vivo identification of the protein's interactors. Here, we show that XAB2 is a partner of the core spliceosome, through its interaction with the PRP19 complex. Ablation of XAB2 results in defective splicing as shown by extensive intron retention, suggesting that XAB2 is indispensable for proper pre-mRNA processing. Moreover, we show that XAB2 binds pre-mRNAs and that this binding is lost in the presence of DNA damage leading to accumulation of unspliced pre-mRNA.

Περίληψη

Η ακεραιότητα του γενώματος βρίσκεται υπό τη συνεχή απειλή περιβαλλοντικών και ενδογενών παραγόντων. Προκειμένου να αντιμετωπίσουν την απειλή αυτή, τα κύτταρα έχουν αναπτύξει μηχανισμούς που αναγνωρίζουν τις DNA βλάβες και τις επιδιορθώνουν. Ο μηχανισμός επιδιόρθωσης μέσω εκτομής νουκλεοτιδίων NER, είναι ένα ιδιαίτερα συντηρημένο μονοπάτι, που επιδιορθώνει ένα φάσμα από αλλοιώσεις της διπλής έλικας. Μεταλλάξεις που οδηγούν σε ελαττωματικό NER έχουν συσχετιστεί με καρκίνο και πρώιμη γήρανση, ωστόσο νευρολογικές, αναπτυξιακές και μεταβολικές διαταραχές που παρατηρούνται στα NER σύνδρομα, δεν μπορούν να καταλογιστούν πλήρως στην αδυναμία διόρθωσης, γεγονός που υποδεικνύει ότι οι παράγοντες του NER, έχουν επιπλέον λειτουργίες πέρα της επιδιόρθωσης του DNA. Πράγματι, πλέον γνωρίζουμε πως οι παράγοντες του NER εμπλέκονται στην μεταγραφή, αναδιάταξη της χρωματίνης και πιθανά στο μάτισμα του πρώιμου mRNA (pre-mRNA). Η πιθανότητα συσχέτισης μεταξύ επιδιόρθωσης/απόκρισης σε βλάβες του DNA και του ματίσματος του pre-mRNA, ήρθε πρόσφατα στο προσκήνιο. Έχει δειχθεί πως πρωτεΐνες που συμμετέχουν στο μάτισμα του pre-mRNA, ρυθμίζουν, σε μετα-μεταφραστικό επίπεδο, την ορθή έκφραση γονιδίων που συμμετέχουν στην απόκριση σε βλάβες DNA (DDR) ή ότι στρατολογούνται στην περιοχή της βλάβης. Μία πρωτεΐνη που συνδέει το μάτισμα του pre-mRNA με την επιδιόρθωση του DNA, είναι η XPA binding protein 2 (XAB2), η οποία εμπλέκεται στο NER, στην μεταγραφή και στο μάτισμα, ωστόσο η ακριβή της λειτουργεία δεν είναι γνωστή. Προκειμένου να διερευνήσουμε τη λειτουργική συνεισφορά της XAB2 στη φυσιολογία του ήπατος, ποντίκια με βιοτινυλιωμένη XAB2 παρήχθησαν, ώστε να γίνει εφικτός ο χαρακτηρισμός πρωτεϊνών με τις οποίες η πρωτεΐνη αλληλεπιδρά *in vivo*. Στη συγκεκριμένη μελέτη, δείξαμε πως η XAB2 είναι μέλος του κεντρικού συμπλόκου ματίσματος, μέσω της αλληλεπίδρασης της με το σύμπλοκο PRP19. Απαλοιφή της XAB2 οδηγεί σε προβληματικό μάτισμα, όπως μαρτυρά η εκτενής κατακράτηση ιντρονίων, κάτι που υποδηλώνει πως η XAB2 είναι απαραίτητη για τη σωστή τροποποίηση των pre-mRNA. Επιπρόσθετα, δείχνουμε πως η XAB2 προσδένει pre-mRNA, καθώς επίσης ότι η πρόσδεση αυτή χάνεται παρουσία DNA βλαβών, κάτι που οδηγεί σε συσσώρευση μη-ματισμένων pre-mRNA.

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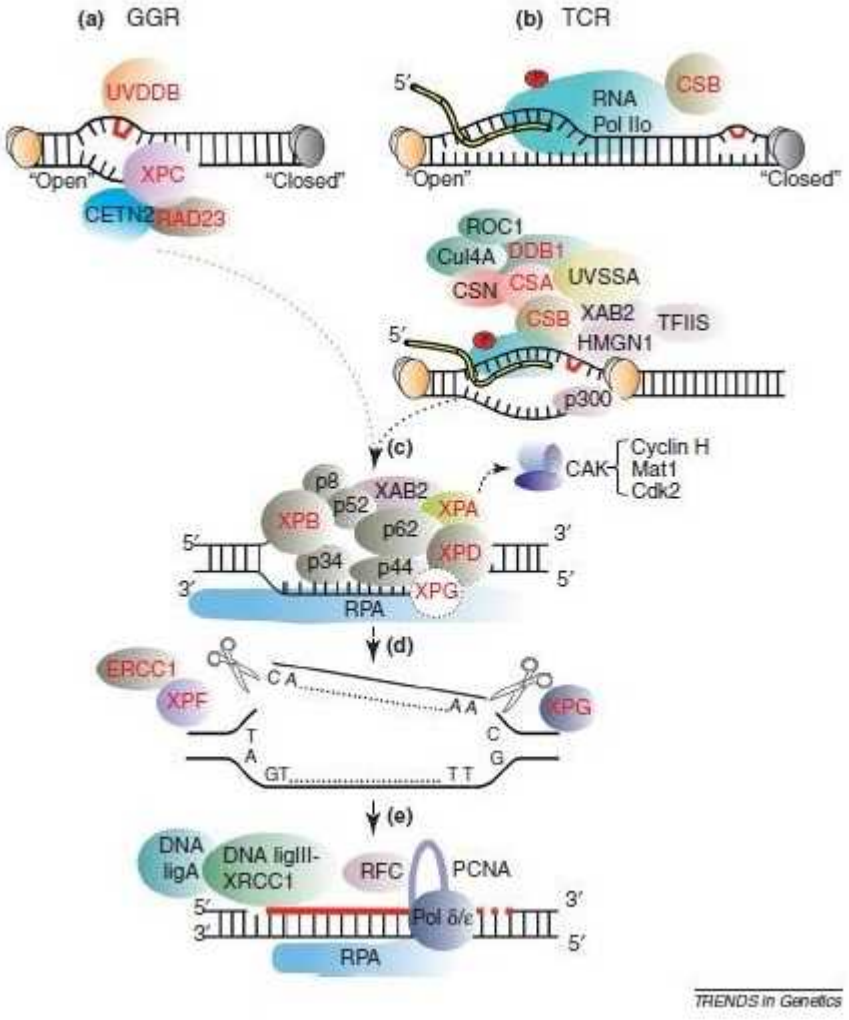
First of all I would like to thank Tsekrekou Maria, for letting me work in her project and for being an inspirational supervisor, always willing to help and provide knowledge. I also want to thank Chatzinikolaou Georgina for all her help and excellent supervision in my side project, during my master thesis. Of course I thank Garinis George for the opportunity he gave me to work in his lab, for all his guidance and valuable advices he provided. Last but not least, all the lab members, who have been really supportive all the way.

Introduction

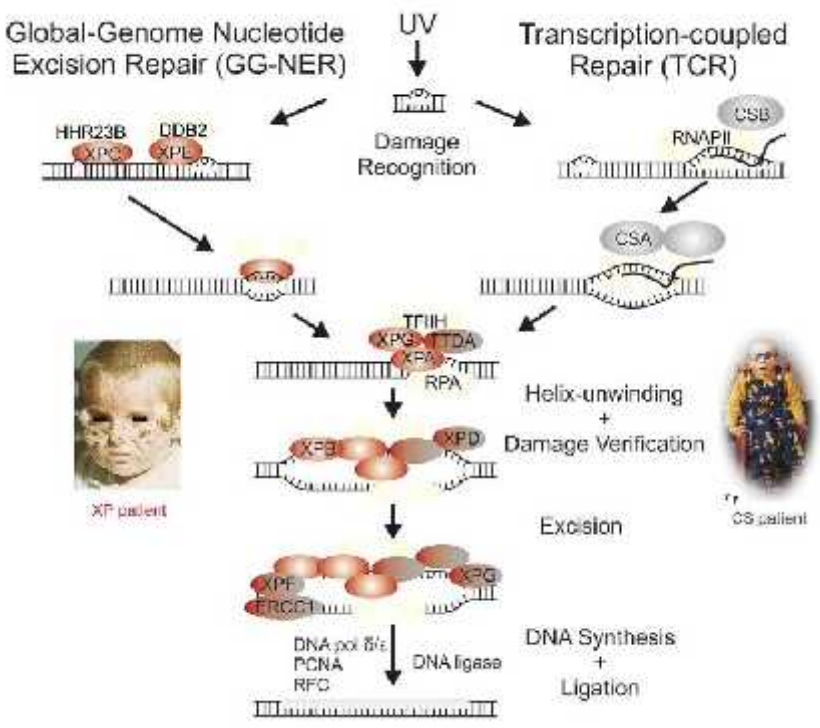
The Nucleotide Excision Repair Pathway

Genome integrity is constantly threatened by exposure to a myriad of environmental agents, or to endogenously formed metabolic products and by-products, replication errors etc[12]. Accumulation of DNA insults is associated with a plethora of diseases ranging from infertility, to cancer and neurodegenerative disorders[3]. Therefore it is of vital importance for the cell to detect DNA damage, signal its presence, and effect DNA repair, cell cycle arrest, and ultimately cell fate decisions, which are together called the DNA damage response (DDR)[3]. To address such insults, mammalian cells have evolved partially overlapping DNA repair systems, in order to counteract structural DNA modifications [e.g. nicks, DNA double-strand breaks (DSBs), etc.] that may obstruct DNA replication or transcription[4]. One major pathway is the evolutionarily conserved Nucleotide Excision Repair (NER) pathway, which removes double helix distorting lesions, including UV-induced cyclobutane pyrimidine dimers and 6/4 photoproducts[5]. NER comprises approximately 30 proteins in two sub-pathways: the Global Genome Repair-NER (GG-NER), which scans the entire genome for helix distortions (that is, disturbed base pairing) associated with structural changes to nucleotides, and the Transcription Coupled-NER (TC-NER), which is initiated when RNA polymerase II (RNA Pol II) is stalled during transcript elongation by a lesion in the template strand[2]. The two sub-pathways differ in the initial steps of DNA damage recognition where they employ different proteins, however both share a common repair machinery. Briefly, DNA is restored in a “cut and paste” manner, by unwinding the double helix, removing a 22-30 base oligonucleotide that contains the damage and filling the single strand gap by synthesis and ligation[2] (Picture 1).

Mutations in at least 13 NER genes, have been correlated to NER-related diseases[6]. In humans, inborn defects of proteins participating in NER, lead to the skin cancer-prone xeroderma pigmentosum (XP) or to a heterogeneous group of premature aging-like (progeroid) disorders, including Cockayne syndrome (CS) and trichothiodystrophy (TTD) [7] [8] (Picture 2). There are also patients with combined symptoms of XP and CS or TTD as a result of defects in the common pathway[9]. It is important to note that the severity of the symptoms may vary, depending not on the affected gene per se but rather on the mutation causing the defect. Mouse mutants with inborn defects in NER closely mimic their human counterparts and display severe developmental abnormalities and shorter lifespan[10].



Picture 1: *An illustration of the Nucleotide Excision Repair Pathway Kamileri et al. 2012*



Picture 2: *NER-associated diseases*
Schumacher, Bioessays 2009

The clinical heterogeneity and varying severity of the features accompanying NER syndromes[7] cannot be fully explained by the DNA repair defect alone, suggesting that NER factors are not constrained in a DNA-repair role only but rather participate in other fundamental processes. Indeed, NER factors have been reported to participate also in the regulation of gene expression[11] [12], chromatin looping[13], the transcriptional reprogramming of pluripotent stem cells[14], as well as the fine tuning of genes associated with growth during postnatal development[15] [16] and possibly pre-mRNA splicing[17].

DNA damage and RNA Binding proteins

Post-transcriptional gene expression is regulated, in large, by RNA-binding proteins (RBPs). RBPs recognize and directly bind specific RNA sequences and secondary structures in pre-mRNAs, mRNAs, or regulatory noncoding RNAs (ncRNAs) [18] [19], to direct capping, alternative splicing and cleavage in the nucleus and stabilization and translation of mRNAs in the cytoplasm. Strikingly, unbiased proteomic approaches, functional screens and studies on individual RBPs have identified RNA processing and/or translation factors, as major functional categories of gene products that are post-translationally modified by DNA damage-signaling proteins[20] [21] and are required for the DDR [2223]. Three major roles have been assigned to RBPs concerning their contribution to genome integrity maintenance: (i) RBPs refine the proteomic complexity required for the DDR through transcriptional and post-transcriptional control of gene expression; (ii) they prevent harmful DNA/RNA hybrids; and (iii) they are directly involved in DNA repair. Consistently, many RBPs are targeted by DNA damage signaling at the levels of post-translational modifications, subcellular localization, and expression[3].

DNA damage tends to globally repress gene expression, which is mainly achieved through a decrease in the levels of mRNAs and mediated through several mechanisms[3]. For example, DNA damage triggers the repression of pre-mRNA 3'-end processing [24],[25] affecting the stability of mRNAs. It is not known whether and how DNA damage affects splicing globally, however splicing of specific pre-mRNAs is altered. In particular, , DNA damage results in a decrease in the expression of functional gene products by switching alternative splicing towards variants harboring premature stop codons, which are subject to nonsense-mediated mRNA decay (NMD) [26] [27]. Beyond inhibitory actions at the pre-mRNA level, DNA damage leads to a decrease in the stability of many mRNAs [28] and to an inhibition of the activity of general translation factors involved in initiation [29] and elongation [30]. The DNA damage-induced decrease in mRNA stability and translation is more widespread than transcriptional repression [28] [31] [3].

Pre-mRNA splicing and DNA damage

Eukaryotic genes contain protein coding (exons) and non-coding (introns) sequences which are both enclosed in the primary transcript. The spliceosome machinery removes introns that disrupt the protein coding sequence to generate mature mRNA which will be translated to functional protein[32] [33]. The major spliceosome (U2 spliceosome) is a highly dynamic complex comprising the five small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4, U5 and U6 named after the respective U snRNAs[34] [35] and is responsible for the excision of more than 99% of human introns. The minor spliceosome

(U12 spliceosome) consists of the U5 snRNP, and the functional analogues of the major spliceosome U11, U12, U4atac and U6atac snRNPs[36]. Spliceosomal proteins, apart from regulating alternative splicing decisions upon DNA damage, have been also shown to translocate at the site of damaged DNA. For example, upon UV irradiation members (PRP19, BCAS2) of the core spliceosomal PRP19 complex translocate from the spliceosome to the site of damage to assist in ATR activation[37].

In this topic, XPA Binding Protein 2 (XAB2) which has long been reported to be part of the TC-NER pathway, as well as to participate in transcription[38], has also been implicated in pre-mRNA splicing[17]. XAB2 belongs to the tetratricopeptide repeat (TPR) superfamily and comprises 15 TPR domains throughout the protein molecule[38 39]. The TPR superfamily includes proteins with divergent functions such as cell cycle control, transcription regulation and RNA processing. Co-immunoprecipitation experiments showed that XAB2 interacts with RNA-polymerase II and NER factors after UV irradiation of HeLa cells, implying its involvement in transcription and TC-NER[17]. The same study also showed that pre-mRNA splicing factors (hAquarius, hPRP19, CCDC, hISY1, PPIE) are partners of the protein. In gel mobility shift assays the protein specifically interacts with RNA but not DNA, which further enhances the assumption that XAB2 might be involved in pre-mRNA processing. The protein's yeast homologue SYF1 is found to participate in the pre-mRNA splicing process (interacts also with Prp19 and Isy1), be essential for the TREX occupancy at transcribed genes[40] and additionally be involved in the cell cycle control, as SYF1 mutants arrest in the G2/M checkpoint[41 42]. Taking into consideration that transcription, pre-mRNA processing and mRNA export are tightly connected[43], it seems possible that XAB2 may be a link between these processes. Moreover, Tanaka and colleagues tried to generate a knockout mouse model but eliminating the gene results in embryonic lethality: embryos die after the morula stage before blastocyst formation and implantation[39]. Here we show that XAB2 is an essential partner of the core spliceosome. Moreover, XAB2 ablation as well as DNA damage results in intron retention.

Materials and Methods

Cell culture

Hepatoma cell line (HEPA) was cultured in DMEM supplemented with 10% FBS and 1% PSG. Culture conditions were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For co-immunoprecipitation experiments, cells were subjected to 20J of UV irradiation and left for 1 hour to recover. For qPCR assays, the cells used, were subjected to 20J UV irradiation and allowed to recover for 6 or 12 hours, before they were harvested. Knockdown experiments were conducted by using JetPrime (Polyplus) transfection reagent according to the manufacturer's protocol and at a 50nM final concentration of dsRNA.

Nuclear Extraction Protocol

1. Tissues:

Liver samples were defrosted in ice-cold Sucrose A buffer (0.32M sucrose, 15mM Hepes pH7.9-0.1% NP40, 60mM KCl, 2mM EDTA, 0.5mM EGTA, 0.5% BSA). Next they were homogenized until no tissue fragments were visible. Cells were pelleted and washed with ice-cold PBS supplemented with protease inhibitors.

2. Cells:

Cells were detached with 0.5% trypsin-EDTA and the pellet was washed with ice-cold PBS supplemented with protease inhibitors.

To obtain the cytoplasmic protein fraction, cells were lysed by NP-40 lysis buffer (10mM Tris-HCl ph 7.4, 10mM NaCl, 3mM $MgCl_2$, 0.5% NP-40, 1mM PMSF). Soluble nuclear proteins were subsequently extracted by incubation of the nuclei with High Salt buffer (10mM HEPES pH7.9, 380mM KCl, 3mM $MgCl_2$, 0.2mM EDTA). Protein concentration was determined by Bradford assay.

Immunoprecipitation/Pull Down with Strep Beads

Beads were equilibrated and then blocked with HENG buffer (10mM HEPES-KOH pH 9, 5mM $MgCl_2$, 025mM EDTA, 20% Glycerol)-200mg/ml Chichen Egg Albumin. Nuclear Extracts (400-600µg) were diluted 1:3 with HENG buffer in the presence of benzonase and RNase and incubated overnight at 4°C to allow sufficient degradation of nucleic acids. Next, nuclear extracts were adjusted at pre-cleared with 100µl beads and subsequently incubated overnight at 4°C with antibodies or species-specific normal serum. Complexes bound to antibodies were pulled-down by incubation with 50µl of pre-equilibrated and blocked beads for 2 hours at 4°C. Beads were subsequently washed with Wash Buffer (HENG, 380mM KCl, 0.3% NP-40)and eluted by boiling in 30µl 1x Laemlli Buffer at 80°C for 10min. Pull down with streptavidin beads was performed in a similar way, except that the preclearing step was omitted.

Details for the antibodies used per IP (400-600µg of protein extract) are as follows: 5µg of rabbit a-XAB2 (Abcam), 2µg rabbit a-BCAS2 (Novus), 5µg rabbit a-PRP19 (Abcam), 3µg rabbit a-DDB1 (Bethyl), 5µG rabbit a-AQR (St John's Laboratory), 5µg normal rabbit serum (Santa Cruz).

Western blotting

Protein extracts were separated by SDS-PAGE and transferred onto Nitrocellulose membranes. The bands were visualized using Ponceau S, blocked with 5% non-fat milk and blotted with various antibodies.

Reverse Transcription

RNA Template	1µg
Oligo dT	1µl
RNase free water	Up to 11µl

- Incubate at 65 °C for 10'

- Cool at 4 °C for 5’ and add the following mix:

5xRT buffer	4µl
10mM dNTPs	1µl
0.1M DTT	2µl
RNase OUT (40u/µl)	1µl
RT enzyme	1µl

- Proceed by incubating at 42 °C for 60’
- Cool at 4 °C
- Dilute samples 5x (add 80µl ddH_2O) and store at -20 °C

Incubations were done in a PCR machine.

Real-time polymerase chain

The sequences of the primers used are:

Nop56	Ex8_F	GTACAGATCCTTGGGGCTGA	ATM	IN60_F	TGGCTGCATCTACCTGTGAC
	Ex9_R	ATACGGCCTTTGTTCTTTGC		IN60_R	TGCTAGTCAGCCCTCCTCAT
	In8_R	CCCCAAACAAAACAGTGGAC		Ex60_F	TACATCCTTGGACTTGGCGA
				Ex60_R	ACCCAGGTCTATGTGCACAA
Utp15	Ex8_F	CGTCCACAGCTTTGACTACG		Ex61_R	CCCCTGTTCAAAAGCCACTC
	Ex9_R	GCTTCAGACTTCCGGTGTTT			

	In8_R	CCAAAGGTAGAGCCCAGAGA	ERCC1	IN6_F	CTAAGCCAAGCATGGTGACA
				IN6_R	TGGGGAGAACAGAACAAACC
Cdk9	Ex2_F	TAAAGCCAAGCACCGTCAG		Ex6_F	AAAGATCCCCAGCAGGCTC
	Ex3_R	TTGATTTCCCTCAAGGCTGT		Ex7_R	ATAAGGAGGTCGGCTGGCTT
	In2_R	CCCTTTGAAGTAGCGGTGTG			
			CDK7	IN10_F	TTTCTCGTGTCATCCTGGCT
Ddit3	Ex2_F	TGTTCCAGAAGGAAGTGCATC		IN10_R	TTAAGCACTTGGGAGGCAGA
	Ex3_R	ACACCGTCTCCAAGGTGAAA			
	In2_R	CAAAGCAACATGAGCAGCAT		Ex10_F	ACTGCAGCACATCTTCATCG
				Ex11_R	GGGGCGGTTACTGAAGTACT
Rbm28	Ex6_F	GCTGTGGCAAAGGACAAATA		Ex10_R	CTGTGAGGCTGTGGTCCG
	Ex7_R	TTCCACCCTGCAGTTCTTCT			
	In6_R	CCCTCACAACCCTCCTACCT	RAD9a	Ex1_F	GTAGTAGCTGCTGGGACTCA
				Ex2_R	TTCGGGATAGCGAATGGACA
Aurkb	Ex7_F	TGAGACGTATCGTCGGATTG			
	Ex8_R	GTTGCCAGGGGTTATGTTTG		IN1_F	GGCAACGTGAAGGGTAAGTT
	in7_R	CATTTTTGCAGAGCCGTGTA		IN1_R	CGGGGAGGAGAACAGAAAGT
Cenpa	Ex1_F	CAAGGAGGAGACCCTCCAG	ATM	Ex3_F	TATCAGCCACCACACAGAGC
	Ex2_R	TTCTGTCTTCTGCGCAGTGT		In3_R	ACACGCAGCCAAGGTTACTT
	In1_R	ACCAGGAACCGTCCCTCTAC		Ex4_R	CAAGAGGTCTTGACATTTTAGCC
Terf2ip	Ex1_F	CCTCCCAGAAGCTCAAACG	ERCC1	Ex3_F	GTGTGCTGCTGGTTCAAGTG

	Ex2_R	CACTCCTCCTCAGGCAAGTC		In3_R	CATTCTGCACTGAGGTCCTG
	In1_R	CAACAGAGAAGAGGGGCTGA		Ex4_R	GGTGCAGTCAGCCAAGATG
Aaas	Ex5_F	GATTGCTGAATTTGCCCAAG	c-FOS	Ex3_F	ATCCGAAGGGAACGGAATAA
	Ex6_R	CCACTGCAAACTTGTTGGTG		In3_R	CCATAGTCCCAAGCTCCAAC
	In5_R	AGGAGTTTTGGCCTTCACCT		Ex4_R	CCTTCTCTTTCAGCAGATTGG
Nup88	Ex11_F	GAGTCTCCACTGCGCATTCT	KIN17	Ex4_F	ATACATCAGCCACCGAGAGC
	Ex12_R	GGAGCCAAATCCTTTTCAGA		In4_R	ATGACGGCTCCCCTAGAAAC
	In11_R	GCCATAAGTTCAATCCCCATT		Ex5_R	CGGATGGTTTCTGGGTCTCT
ERCC2	Ex20_F	GGCTGTGATCATGTTTGGAG	DDB1	Ex1_F	AGTGAACGGCTGTGTGACC
	In20_R	CCCTGACAGCACTGTTTCC		In1_R	GTGGGAACAGCTCCTTTGAG
	Ex21_R	CAGCAAAGACCATGAGTCCA		Ex2_R	GCCTGAAGAGCTCCATGACT
Pold1	Ex23_F	GCTCTTGCGCATCTTTGAG		Ex15_F	CCTCGATCCATCCTGATGAC
	Ex24_R	GTCTTGCATCGTGTGTGGTC		In15_R	AGCTGCTTGGTTAAGGCTCA
	In23_R	CGCTCACACTCCCTCCTG		Ex16_R	AGAGCAAGCAAAGACGTTGG
DDB2	Ex5_F	AATCTGATCTCCCACCCTCA			
	In5_R	CCCCTTCCCATAATGACAGA			
	Ex6_R	GGTATCGGCCCACAACAAT			

qPCR mix:

5x diluted cDNA	2.5µL
50mM MgCl2	1.25µL
10x Platinum Taq Buffer	2.5µL
SYBR1 (1/2000)	0.75µL
10mM dNTPs	0.4µL
qPCR primer mix	4µL
H2O	13.5µL
Platinum Taq Polymerase	0.1µL
Final volume	25µL

qPCR Program:

1)94 °C	5’
2)94 °C	15’’
3)60 °C	25’’
4)72 °C	25’’
5)78 °C	1’’
6)Repeat steps 1-5, 40 times	
7)72 °C	2’
End	

Results

In order to investigate the functional contribution of XAB2 during mammalian development biotin-tagged XAB2 mice were generated. Briefly, XAB2 was fused with an affinity purification tag, comprising an avitag sequence and a 3X FLAG tag. Homozygous aviXAB2$^{+/+}$ mice were crossed with mice expressing the BirA biotin ligase transgene, which specifically recognizes and biotinylates the Avitag, creating a high affinity "handle", which in turn allows one to isolate XAB2-bound protein complexes from biotin-tagged XAB2 (bXAB2) mice by binding to streptavidin coated beads. All the necessary tests were carried out, in order to ensure that the biotin-tag does not interfere with embryonic and postnatal murine development or with DNA repair efficiency (data not shown).

In order to identify XAB2-associated protein complexes present in hepatic tissue, the in vivo biotinylation tagging approach was combined with unbiased high-throughput proteomics. To do this, nuclear extracts from P15 bXAB2;BirA livers and livers expressing only the BirA transgene were prepared, implementing high-salt extraction conditions. Nuclear extracts were treated with benzonase and RNase, to rule out any protein interactions mediated by DNA or RNA, and subsequently with streptavidin-coated beads. Next, the pulled-down proteome was separated by 1D SDS-PAGE followed by in-gel digestion and peptides were analyzed with high-resolution liquid chromatography-tandem mass spectrometry (nLC MS/MS) on a hybrid linear ion trap Orbitrap instrument (data not shown). Three biological replicates shared 645 proteins, which were subjected to gene ontology (GO) classification. Those biological processes (Fig. 1A) or pathways (Fig. 1B) containing a significantly larger than expected number of proteins relative to the murine proteome, were defined as significantly over-represented (FDR<0.05). At this confidence interval, mRNA splicing is highly enriched in both biological processes and pathways suggesting that XAB2 might function mainly in splicing. Moreover, a number of DNA repair factors co-precipitated with XAB2, implying an additional role in DNA damage repair/response.

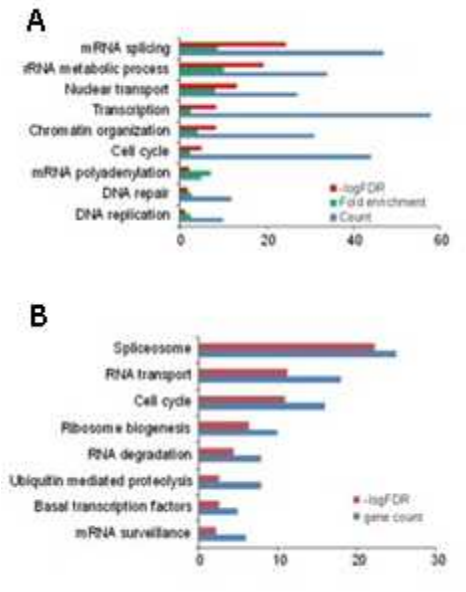


Figure 1: *A,B, List of significantly over-represented biological processes or pathways respectively.*
**(Unpublished data)*

XAB2 is part of a spliceosomal complex

We tried to validate the interactions between XAB2 and some of the proteins involved in the aforementioned biological processes. We selected the NER factor DDB1 and the spliceosomal factors PRP19, BCAS2 and AUARIUS (AQR). Briefly, DDB1 which is required for DNA repair, binds to DDB2 to form the UV-damaged DNA-binding protein complex (the UV-DDB complex), that may recognize UV-induced DNA damage and recruit NER factors to initiate DNA repair. It is also associated with protein ubiquitination[44 45]. PRP19 which is a ubiquitin-protein ligase and is a core component of several complexes mainly involved in pre-mRNA splicing and DNA repair. More specifically it is a core component of the PRP19C/Prp19 complex/NTC/Nineteen complex which is part of the spliceosome and participates in its assembly, its remodeling and is required for its activity[46 47 48 49 50]. BCAS2 which is a component of the PRP19-CDC5L complex that forms an integral part of the spliceosome and is required for activating pre-mRNA splicing. It may have a scaffolding role in the spliceosome assembly as it contacts all other components of the

core complex[48,51,52]. Finally, AQR is involved in pre-mRNA splicing as component of the spliceosome. It is an intron-binding protein with an ATP-dependent RNA helicase activity[52–56] and is required to link pre-mRNA splicing and snoRNP (small nucleolar ribonucleoprotein) biogenesis. It may act by helping the folding of the snoRNA sequence. It binds to intron of pre-mRNAs in a sequence-independent manner, contacting the region between snoRNA and the branchpoint of introns during the late stages of splicing.

By using pull down with streptavidin bead of nuclear extracts from P15 bXAB2;BirA livers and livers expressing only the BirA transgene, we were able to validate that bXAB2 interacts with all the splicing-related proteins, namely BCAS2, PRP19 and AQR (Figure 2A). Under these conditions, we could not find a consistent interaction with DDB1. To further investigate whether this interaction would be enhanced upon DNA damage, we used nuclear extracts form HEPA cells subjected to UV. Under these conditions, we also assessed whether the spliceosomal complex remains intact. Thus, HEPA cells were either untreated or subjected to 20J of UV irradiation and left for 1 hour to recover. Nuclear extracts were then subjected to immunoprecipitation (IP) with antibodies raised against XAB2, BCAS2, PRP19 or DDB1. Unfortunately, IgG controls showed a very high background, we tried, however, to interpret the results given that some of the interactions have been verified in untreated conditions by streptavidin pull down. Thus, XAB2 interacts with BCAS2 interaction both in untreated and UV irradiated cells (Figure 1B), which was observed in a reciprocal way (Figure 2B, D). XAB2 seems to interact with PRP19 but this interaction has not been verified in a reciprocal manner due to possible failure of the antibody against PRP19 (Figure 2C). In addition, it seems that XAB2 and DDB1 interact in UV irradiated cells, but the absence of a band in input and DDB1 IP lanes suggests that this finding might be unspecific (Figure 2E). Moreover, XAB2 seems to interact with AQR upon UV irradiation (Figure 2F). Our immunoprecipitation experiments need to be further standardized in order to be able to answer whether the spliceosomal complex remains intact upon DNA damage and if a UV-mediated interaction with DDB1 exists. To summarize, the pull down experiments that present much less background, provide a strong indication that XAB2 is part of the core spliceosome.

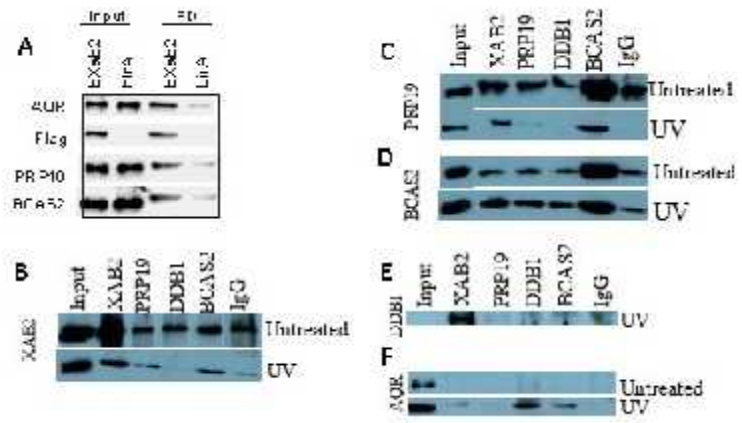
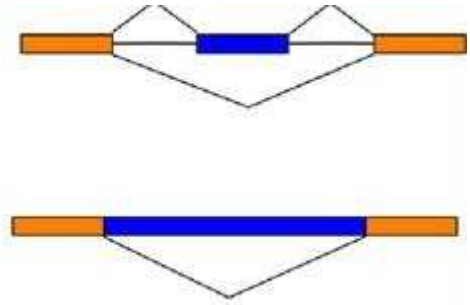
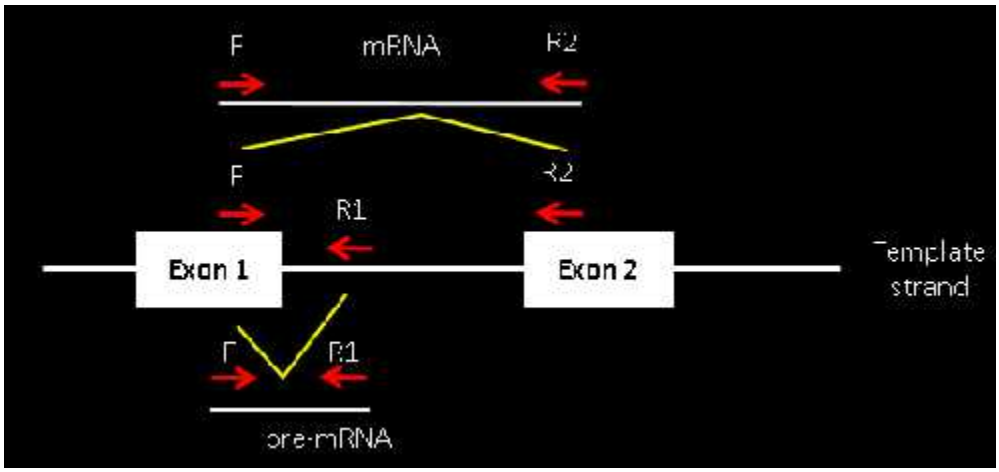


Figure 2. ***XAB2 is part of a spliceosomal complex*** *A, Endogenous bXAB2 from P15 livers was pulled down in order to confirm its interaction with Aquarius, PRP19 and BCAS2. Protein extracts from birA livers were used as a control for unspecific interactions, B-F, Co-immunoprecipitation assays to further validate that XAB2 interacts with AQR, PRP19, BCAS2 and DDB1. B, Blot against XAB2 (1:2.000). XAB2-BCAS2 interaction is observed only upon UV. C, Blot against PRP19 (1:1.000). PRP19 interacts with BCAS2 and upon UV it is also associated with XAB2 and PRP19. D, Blot against BCAS2 (1:5.000). Only upon UV treatment we observe BCAS2 interacting with XAB2, PRP19 and DDB1. E, Blot against DDB1 (1:5.000). XAB2 precipitates DDB1 upon UV treatment. F, Blot against AQR (1:500). AQR associates with XAB2, DDB1 and BCAS2 upon UV.*



Picture 3. *A schematic of intron retention. A sequence may be spliced out as an intron or simply retained. This is distinguished from exon skipping because the retained sequence is not flanked by introns. If the retained intron is in the coding region, the intron must encode amino acids in frame with the neighboring exons or a stop codon or a shift in the reading frame will cause the protein to be non-functional. This is the rarest mode in mammals (Sammeth et al. 2008). Picture from: Zhao et al. 2010*



Picture 4. *An illustration of how our qPCR primers hybridize with the template strand in order to monitor the amplification of transcripts with their intron retained or not.*

XAB2 ablation and UV-induced DNA damage result in intron retention

XAB2 ablation has been associated with alternative splicing events[17 51], however the impact on gene expression genome wide has not been yet investigated. To address this, the effect of XAB2 knock down on splicing events of HEPA cells was examined, by performing mRNA sequencing. The majority of the splicing decisions upon XAB2 abrogation, were related to intron retention (IR), indicating that XAB2 is essential for proper splicing. The majority of the transcripts with intron retention, which we selected for validation, are involved in DNA damage and splicing. Indeed, accumulation of such transcripts was observed at either 48h or 72 h or at both time points following XAB2 knockdown (Figure 3A-B). We find three trends: (a) accumulation of intron retention at 72h combined with increased mRNA levels (Ddb1, Ercc2, Pold1, Utp15, Aurkb, Nup88), (b) intron retention at 48h but absence at 72h combined with severely decreased mRNA levels at this time point (Nop56, Ddit3, Rbm28, Cenpa, Terf2ip, Aaas), (c) relatively stable pre-mRNA and mRNA levels. The first category possibly suggests that transcription of the respective genes is increased in order to bypass defective splicing. Upregulation of these genes may as well be necessary to respond to the deficient processing machinery. The second category presents with much higher intron retention at 48h and is typical for intron retainment. The respective transcripts are severely downregulated at the mRNA level, suggesting that any attempt for their transcription is

abandoned. Finally the last category implies that these genes have a very low turnover. We have to note that these experiments have been performed only once and need to be further validated; in addition we use transient transfection which always results in residual Xab2 expression, thus we cannot fully abrogate XAB2 and possibly the spliceosome functionality.

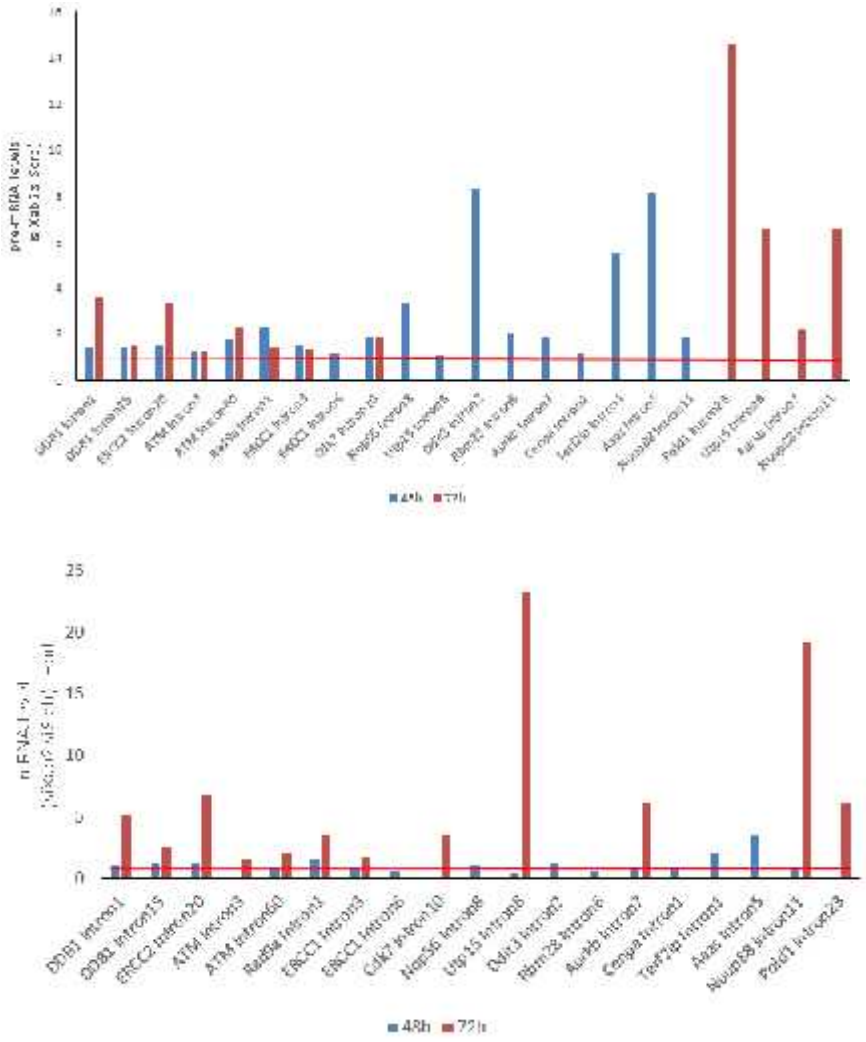


Figure 3. ***XAB2 ablation and UV-induced DNA damage result in intron retention***

HEPA cells were treated with dsRNA targeting the XAB2 transcript or with siScrb (control) and after the indicated time point (48 or 72 hours), they were collected. Total RNA was isolated and reverse transcribed. A To calculate the pre-mRNA levels, we first compared the CT values of each transcript containing an intron to the corresponding intron-free transcript, both for siXAB2 and siScrb treatments and then we compared the resulting value upon siXAB2 over the respective value upon siScrb treatment. More transcripts retained their introns after 48h than after 72h B Moreover we calculated the amount of mRNA, by comparing the CT value of each mRNA to the CT value of HPRT (control gene), both for siXAB2 and siScrb treatment and the relative mRNA levels were calculated by comparing these two values.The red line denotes value equal to 1 that is the baseline (siScrb). Intron number indicates that the primers were designed at the adjacent exons.

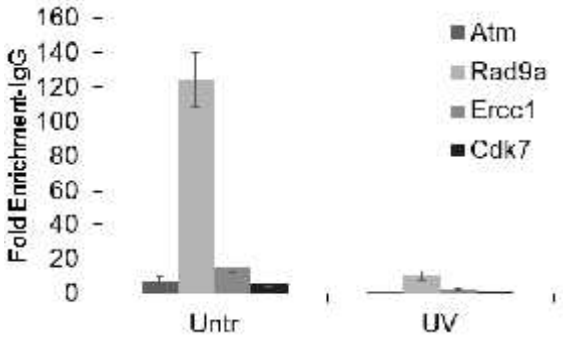
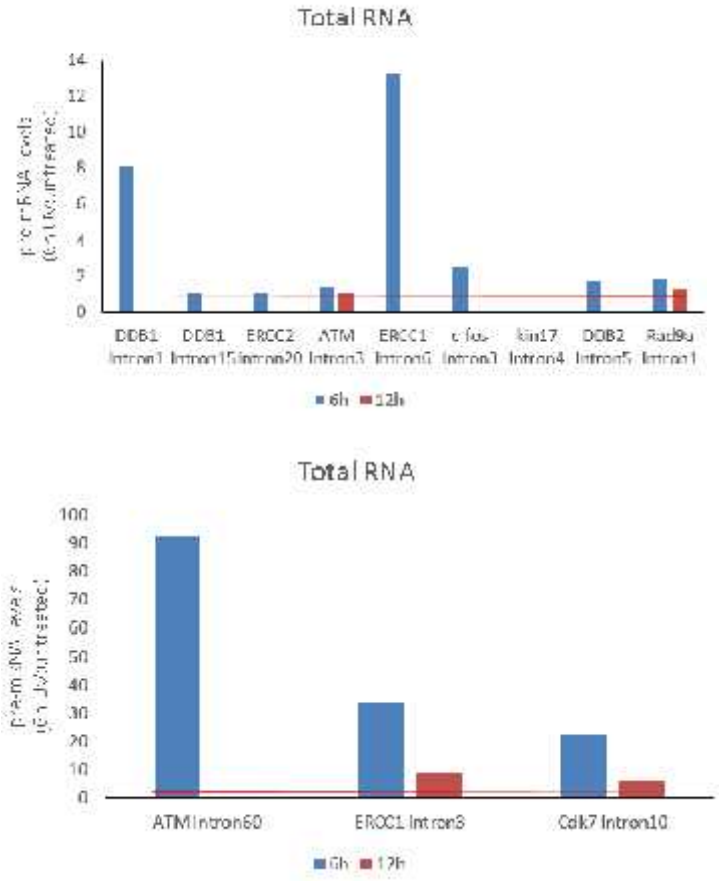


Figure 4. *RNA immunoprecipitation was performed to test whether XAB2 retains its ability to bind pre-mRNAs of selected genes or if this association is abrogated upon UV irradiation. RIP was performed on nuclear extracts from HEPA cells.*



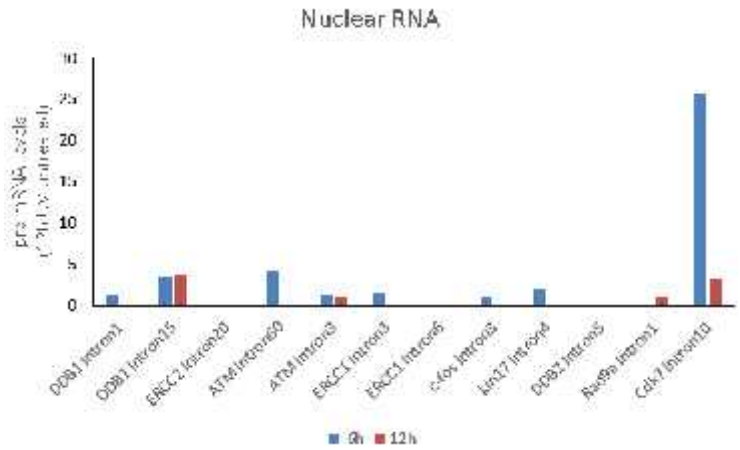


Figure 5: XAB2 ablation and UV-induced DNA damage result in intron retention

HEPA cells were subjected to UV irradiation and allowed to recover for 6 or 12 hours, before they were collected. Total and nuclear RNA was separately isolated and reverse transcribed (5A-B and 5C respectively). The qPCR analysis was conducted as described in Figure 3, with the difference that in this case we compared the values from UV-treated cells, over the ones that belong to the untreated cells. As shown, transcripts with their intron retained are more enriched in the cases of total mRNA. What is more, at 72h in both nuclear and total mRNA, all the transcripts are part of the ones enriched at 48h post-UV but with a few exceptions they show less accumulation. The reason we chose to work with nuclear and total mRNA is because the levels of spliced mRNA, have been proven to be higher in the nucleus than in the cytoplasm[57].

**The red line denotes value equal to 1*

XAB2 was verified to bind on pre-mRNAs of the selected genes by RNA immunoprecipitation (RIP was performed as described in this publication[58]); this binding is disrupted upon UV-irradiation (Figure 4). In cohort to this, it has been suggested that transcription blocking DNA lesions, promote spliceosome displacement[59]. This evidence led us to validate if splicing is affected upon DNA damage in our experimental setting. We used HEPA cells non-irradiated or UV irradiated and assessed intron retention at 6h or 12h post irradiation. Indeed, pre-mRNAs are accumulated 6h post UV irradiation in all transcripts tested, while this phenomenon seems to decline at 12h post irradiation with the exception of a few transcripts (Ddb1, Cdk7) (Figure 5A-C).

Conclusions

Our work demonstrates that XAB2 is part of the core spliceosome and functions as an essential pre-mRNA splicing factor since its ablation results in defective splicing characterized by increased intron retention. The exact role of XAB2 however in splicing is not clear: we do not know whether XAB2 is necessary for the assembly of the PRP19 complex and whether the latter exists upon XAB2 abrogation. Moreover intron retention was also observed upon UV-induced DNA damage. The low levels of pre-mRNA at 12h post UV, could be explained by the fact that it is possible that most of the damaged DNA has been repaired by that time, while this might not be the case at 6h post UV irradiation, where major pre-mRNA accumulation was observed. We believe that intron retention upon DNA damage, suggests that XAB2 in complex at least with BCAS2 and AQR is displaced from the spliceosome leading to intron retention. Whether the complex is recruited at the site of damage as part of NER remains elusive. More work needs to be done in order to elucidate the exact role of XAB2 during pre-mRNA splicing and DNA damage, as well as to shed light into how these two processes co-exist and associate.

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Annex

Nuclear Extract Preparation (High Salt or RIPA)

Isolate Nuclei from adherent cells

1. Detach cells with 1x trypsin-EDTA (0.05%), spin @ 1000-1100 rpm 5min, RT.
2. Remove supernatant and resuspend pellet in 5-10 ml ice cold PBS (sterile) + 1mM PMSF (i.e for 1 ml pellet, wash with 5 ml PBS) (must see no clumps at all) and spin @ 1000 rpm for 5 min, 4^{o}C.
3. Discard supernatant, again rinse with ice cold PBS 1x- 1 mM PMSF and spin at 1000 rpm for 5 min at 4^{o}C.
4. Resuspend pellet in 5x CPV NP-40 lysis buffer-1mM PMSF, do not vortex. Incubate on a rotating platform at 4^{o}C for 10 min.
5. Cf @ 1000 rpm for 5 min, 4^{o}C. Optimal lysis should result in a whiter nuclear pellet than before. This step removes endogenously biotinylated proteins that are mostly cytoplasmic. Optional: Keep supernatant as cytoplasmic protein fraction.
6. Wash with NP40 lysis buffer and spin strictly at 1000 rpm for 5 min, 4^{o}C.
7. Discard supernatant, resuspend pellet in 5x CPV High Salt or NPV RIPA-like buffer + 1 mM PMSF + 1x PIs cocktail + 1 mM DTT, using a blue tip (not cut) and vortex (~15s).
8. Incubate for 60 min on a rotator at 4^{o}C.
9. Centrifuge to pellet insoluble fraction, 13000 rpm for 30 min at 4^{o}C.
10. Determine the protein concentration by Bradford assay.
11. Nuclear extracts can be snap frozen in liquid nitrogen and stored at -80 ^{0}C. Alternatively proceed to IP/PD.

Isolate Nuclei from mouse tissues

1. De-frost tissue sample in ice-cold Sucrose A buffer.
2. Homogenize for 30s. Repeat if necessary until no tissue fragments are visible.
3. Pellet cells at 1100-1200 rpm for 7-15 min at 4^{o}C.
4. Wash once or twice with ice cold PBS (~10mL), Pellet cells at 1000rpm, 5min, 4^{o}C

5. Continue extraction (from step 4) as above mentioned (perform NP-40 lysis step once).

Buffers

Sucrose A

0.32M Sucrose	0.5mM spermidine (binds and precipitates DNA)
15mM Hepes pH 7.9 0.1% NP40	0.15mM spermine (binds and precipitates DNA from low salt aqueous buffers)
60mM KCl	1mM DTT
2mM EDTA	1mM PMSF
0.5mM EGTA	0.5mM aprotinin (only for livers)
0.5% BSA	1x PIs (stock 100x)

NP40 Lysis buffer

10 mM Tris-HCl pH 7.4
10 mM NaCl
3mM $MgCl_2$
0.5% NP-40
1 mM PMSF

High Salt buffer

10 mM HEPES pH 7.9	20% Glycerol
380 mM KCl	1x PIs
3 mM $MgCl_2$	1mM PMSF
0.2 mM EDTA	1mM DTT

RIPA buffer

50mM Tris pH 7.5	1mM EDTA
1% NP-40	10% glycerol
0.25% Na-DOC	1mM DTT
150mM NaCl	1mM PMSF, 1x PIs

PULL DOWN WITH STREP BEADS

Nuclear Extraction from Adherent cells

1. Trypsinize cells with 1x Trypsin-EDTA

2. Deactivate trypsin with complete medium
3. CF @1000rpm for 5min, RT
4. Wash cell pellet with PBS-1mM PMSF
5. CF @1000rpm for 5min, 4°C
6. Add 3-6CPV (5) of NP-40 Lysis Buffer-1mM PMSF and mix by pipeting
7. Incubate on ice (4°C) for 10min
8. Cf @1000rpm, 5min, 4°C
9. Keep S/N (it is the cytosolic extract)
10. Rinse pellet with NP-40 lysis buffer
11. Cf @1000rpm, 5min, 4°C
12. Add 2-5NPV (5) Nuclear lysis buffer-1mM PMSF-1x PIs-1mM DTT
13. Resuspend pellet by vigorous pipetting and vortexing
14. Incubate @4°C for 1h
15. Cf @13000rpm, 20min, 4°C
16. Determine protein concentration of extracts by Bradford assay
17. Dilute 1:3 the amount of Nuclear Extract needed for pull down with HENG buffer-1x PIs-1m MPMSF
18. Add: 1µL/mg protein Benzonase, 0.2µg/µL RNAse, 2mM $MgCl_2$

Preparation of beads

1. Rinse beads 2x with 1mL PBS
2. Block beads with 200 µg/ml Chicken Egg Albumin in HENG buffer, 1 hour, RT, on a rotating platform.

Pull down

1. Add beads to the treated NEx
2. Incubate O/N @4°C on a rotating platform
3. Remove flow through and keep a small aliquot to test on WB
4. Wash beads 5x in Wash buffer: 2 times fast, 3 times by incubating 10 min on a rotating platform at 4°C
5. If SDS-PAGE analysis is the following step, wash once quickly with 1x PBS to remove salts
6. Elute proteins in 1x Laemmli sample buffer, boil @80°C for 10min

DNA and RNA extraction of NExs and benzonase/RNAse treated NExs

(Check if bensonase and RNase work)

1. Take 40µL NEx and 120µL benzonase/RNAse treated NEx. Bring NEx to 120µL by adding 80µL H_2O
2. Add 1V (i.e. 120µL) Phenol/Chloroform/Isoamylalcohol (25:24:1) and mix well
3. Cf @13000rpm, 5-7min, RT
4. Transfer the upper phase to a new tube and add 1V (120µL) Isopropanol. Mix well
5. Cf @13000rpm, 4^{o}C, 25min
6. Decant S/N
7. Air-dry for 5-10min
8. Add 10µL TE and loading buffer
9. Proceed to agarose gel electrophoresis

Buffers

- HENG Buffer**:** 10mM Hepes-KOH pH 7.9, 1.5 mM $MgCl_2$, 0.25 mM EDTA, 20% glycerol, 1 mM PMSF.
- Wash Buffer**:** 10mM Hepes-KOH pH 7.9, 300 mM KCl, 0.3% NP-40, 1.5 mM $MgCl_2$, 0.25 mM EDTA, 20% glycerol with 1 mM PMSF, 1x PIs (1000x stock)