H3K4ox a New Epigenetic Mark

Characterization of H3K4ox as a new epigenetic mark and its role in chromatin condensation.

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A en Jordi i na Dolors,
els meus pares.

"Mistakes are almost always of a sacred nature. Never try to correct them. On the contrary: rationalize them, understand them thoroughly. After that, it will be possible for you to sublimate them."

Salvador Dalí i Domènech, 1964 *Diary of a Genuis*

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Estic molt feliç d'haver acabat per fi aquest llarg camí del doctorat. Amb il·lusió us volia agrair que m'hagueu fet tant costat sempre. Moltes gràcies de tot cor. Jo en cap cas ho hauria imaginat. Més que res, si he arribat fins aquí és per tots vosaltres. *Parabéns* nostàlgics per a tots. En resum, no canvieu mai. Passaran molts dies, ens distanciarem d'alguns més i d'altres una mica però mai oblidarem el que hem viscut aquest temps. Us estaré agraït per sempre allà on em porti el camí.

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Enroleu-vos que deien, enroleu-vos. Fa quatre anys i escaig, així sense quasi adonar-nos-en, Nos, en Joan Pau vàrem començar una tasca per a la qual no estàvem preparats. Mai s'està preparat del tot, innocents com som, i mai és el moment diuen les novel·les de cavalleresca i com tot en aquesta vida, es tracta d'anar-ne aprenent molt a poc a poc i mentre es fa camí. En aquestes línies aprofitarem oportuns l'avinentesa per tenir un petit detall amb tots aquells qui llur comportament poc o molt ens han ajudat a arribar fins aquí. És tan probable que, oblidadissos com som, ens en quedi més d'un al fons del magí així que d'entrada, no ens ho tingueu en compte.

Encetarem amb un gràcies al *Chromatin Team*.

L'any 2013, i com passa el temps, és a dir fa quatre anys vam iniciar el doctorat en Biomedicina a la UPF de Barcelona amb for-

ça suspens pel tema beca de la mà de na Sandra Peiró i Sales. I decididament, gràcies a na Sandra també l'hem acabat. El doctorat han estat uns anys d'aprenentatge que ens fa difícil descriure apartant les emocions del què han implicat aquests anys sota la seva versada tutela en bioquímica. Si us diem que un laboratori és una espècie de vaixell, na Sandra en seria el capità i els diferents responsables del funcionament del vaixell els doctorands. Mariners àvids d'aventures amb més o menys experiència però grumets al cap i a la fi i amb molt per aprendre. La capitana d'un vaixell anomenat *Chromatin Team*. Com amb tot bon capità no sempre buscàvem el que ens demanava, i a voltes pensaríeu no està sent apropiada o justa però sí que ho era i no hi ha res més difícil que treballar repartint justícia. Han estat cinc anys (un de màster i el propi doctorat) de moments alts i baixos, de rialles i tristos, en essència de amb renys merescuts i felicitacions sornegueres, resumint, anys d'aprenentatge en el sentit més ampli de la paraula. I finalment, ara que acabem aquesta etapa, només ens resta tornar-li incomptables moments de confiança i paciència amb el nostre agraïment. Sota la tutela de na Sandra hem après àmpliament tot el que cal per a ser un professional de la investigació científica i unint-t'ho a la seva passió per la bioquímica hem pogut gaudir d'un exemple impagable en molts aspectes. Així que Gràcies Sandra! Sens dubte hem après amb esforç que la desídia no casa amb el triomf, que les coses ben revestides es fan de constància i que lacerant el caos es té èxit en un món caníbal com és la investigació. Llavors i sempre, tot amanit amb la passió per a la ciència. I tot això, és gràcies a tu.

Recorrent el camí junts, també es mereixen un gràcies gegant gairebé tothom amb qui ens topàrem en aquesta aventura. Els companys del vaixell, altres doctorands amb qui hem crescut en aquest viatge, són els que més ens han aguantat i ajudat en els moments en què ens veiem superats i també els primers en celebrar incomptables instants de joia. Per ordre d'antiguitat i prèvies al determinant enrolament, gràcies, Alba, Ane i Jessica. Amb n'Alba ens coneixíem d'abans i tot i que vam coincidir poc, cadascun dels litúrgics moments compartits sempre fou divertit i amè i decididament poc més demanar en els primers instants d'un grumet o mariner de plana figuerenca com nosaltres. Gràcies i molta sort de cares al nou vaixell en què t'has embarcat des del port de l'IRB. Toca ara n'Ane que fou la darrera d'abandonar la tripulació de la ossada capitana Sandra i una de les persones que més marca ens resta d'aquesta tesi doctoral. Amb els seus alts i baixos, com tot en aquesta vida, sempre serem dos *mindundis* que han après humilment tots amb el seu estils el que cal per ser un bon investigador científic. *Eskerrik asko eta zorte on abentura eta alemanez haize gauzatu hartu duzu*. Na Jessica és sens dubte amb la que més temps ens hem aguantat en la meva estada en el vaixell de la capitana Sandra. Moltes gràcies per tots els moments compartits. Tant moments dels bons com dels no tan bons. Gràcies per totes les aventures insospitades al volant d'una petúnia farcida de peluixos, gràcies per la confiança i gràcies per ser tant generosa.

Llavors, els tres darrers grumets de la tripulació que se'ns van voler unir adés nostre foren na Laura, na Gemma i en Gaetano. *Grazie Gaetano per portare la vostra esperienza sulla barca*, sempre s'agraeix la presència d'un napolità amant del futbol a l'hora de intentar navegar pel mar del doctorat. Gràcies Gemma, i ho dic obertament, per no haver-te destapat com l'olotina orgullosa que ets ultratjar-nos amb el trabuc de ton avi al *lab* i ensenyar-nos com es solucionen les coses envoltats de volcans Deixant de banda les velles bromes per quan et puguem sentir *desputricar* del llegendari olotí, gràcies per aportar les teves ganes de fer les coses ben fetes, l'energia en la feina al laboratori i el donar-ho tot estirada en una inhòspita pista de ball absorta pel ritme del que demana un taxi. Així que molta sort com a grumet més jove del vaixell. Finalment, agrair-li molt a na Laura Pascual. Ella arribà just després nostre i gairebé des del primer moment ens vas fer sentir especials. Recordem sempre revelar *westerns* en vacances i la il·lusió que hi afegia a tot. Vas arribar com un grumet conegut i ara que irremeiablement Nos, en Joan Pau, ens trobem a port esperant que ressalti a l'horitzó el següent vaixell estem molt orgullosos de tot el que hem compartit amb tu. Un grumet amb les idees clares que no únicament hi era sempre sinó quan les onades sacsejaven el vaixell, el feinejar es feia massa feixuc o perdíem el rumb. Gràcies per mostrar-nos la teva personalitat humil i sincera. Gràcies a tota la harmoniosa gent que hem conegut a través teu. Gràcies Adrià per la àmplia part que et toca i gràcies Jack per fer-la tant feliç. Moltes gràcies a tots, sou encantadors i us tindrem sempre molt presents. *Por cierto, nuestra última oferta sigue en pie…*

Un altre gràcies és per a l'*Snail Team.*

En un mar com el del doctorat òbviament no hi ha un únic vaixell. I un que sempre ha navegat molt a prop nostre fou una galera enorme farcida de mariners: La galera *Snail Team* que estudia la transició epiteli-mesènquima des de diferents vessants sota l'atenta i temuda tutela del capità Antonio García de Herreros Madueño. Gràcies de tot cor Antonio per tots els seminaris on ens aportaves els teus assenyats consells i per refregar-nos les victòries del Madrid amb la necessària dosi, ni massa ni poc. Gràcies a tots els membres de la teva tripulació amb els quals als llargs d'aquests anys hem anat coincidint. Cadascú d'ells ens ha aportat alguna cosa i ens ha omplert de records inesborrables. Difícil no deixar-se'n cap. Gràcies sempre als subcomandants: Jepi Baulida per ser tant honest, gràcies també en Víctor Díaz, pel teu sentit de l'humor i amb menys tracte agrair a na Clara i na Sílvia la bonhomia que sempre ens transmetien. De timoner del vaixell hi trobàrem un *vasco*, establert sorneguer a Vilanova i la Geltrú. Moltes gràcies Raúl per totes les ensenyances i els moments compartits. Gràcies als diferents mariners que ara viuen noves travessies més lluny o més a prop: Primer, gràcies Rosa, pels bons moments compartits i per ser la resistent xarxa de seguretat quan començàvem. Gràcies Núria que en res et toca, gràcies Estel, Cristina Figueras, Irene Fuster, Pere Massó i Andrea García perquè poc o molt sempre hi fóreu oportunament per dibuixar un somriure que no sempre és senzill al laboratori. Dels que més recentment partiren, moltes gràcies tant per tu Fresques (Àlex Frias) com per tu Jou. Per tants moments viscuts en els que fèiem el burro, mai ens podrem oblidar del Basquet

SilverBall, *humort* absurd, *xists* dolents, *musicote*, etc. I tot això, generant resultats com uns sonats ho fèieu! De tots dos tinc molt records inesborrables i he après molt. Us desitjo el millor en les aventures noves que us esperen. *Јелена Хвала! Сигурно је добро написан, али сам морао да пробам... Хвала за тако добар савет, речи охрабрења и подршке. Без сумње је то теза би било незамисливо да вас нема шансе да је био део и активна и незаинтересовани само природно на љубазности! Срећно у продавници за нас сутра и да можемо да живимо заједно. Grazie Martina per la vostra gioia innata. Ramona dank u voor het zijn zo vrolijk en de zorg voor details.* I les darreres en marxar: ens cal sens dubte donar gràcies a na Montse i na Meritxell. Gràcies innombrables Txell-Doc per portar-nos el teu bon caràcter i especialment el teu bon humor on tanta falta ens feia. Que tinguis sort per UK. Igualment Lorena! Moltes gràcies per a cuidar el ramat de cabres que eren els grumets de l'*Snail Team* i els que estàvem en el *Chromatin Team* en els darrers mesos.

També tots els membres de la tripulació autònoma. Veure'us ens omplia d'alegria ja que hi deixem a molt bons amics. Per anar tocant a fi aquesta corrua de gent, gràcies a tots els grumets que ara cobreixen les files de la tripulació de l'*Snail Team*, esperem no oblidar-nos a ningú: Aïda, Guillem "*Willy*" Lambies, als *postdocs* Rocco i Santi i els mes nous David "*mi arma*" Cabrerizo, l'*alferez* Jordi i la sergent Rian. Moltes gràcies, *Grazie mille, Muxah graziah en dank u.* Finalment, per complicitat i experiències viscudes, un ostentós esment per en Rubén i na Laura *"Farla"* Sala. Vau arribar el *lab* després nostre i de seguida us hi vau instal·lar com si sempre

n'haguéssiu format part, sols amb la vostra manera de ser i de fer. Costa molt trobar persones de la vostra vàlua personal, i segur que amb la ostensible bonhomia de què feu gala, anirem participant plegats de noves aventures. Podeu estar segurs que us duré en el cor després que, plegats, sobrevisquérem a tantes i diverses aventures com la batalla *pasqualí* amb el *bicho* Víctor. I les darreres setmanes, dir-te gràcies *lehendakària* per a fer-nos tant costat. Hom ha de saber que no hi ha res millor que una ratlla de les teves per oportunament aixecar un matí.

Hi ha un munt de gent a l'IMIM a qui dir gràcies.

A més, la resta de mariners que ens hem anat trobant tant per les utòpiques rutes d'aquest mar del doctorat com en els diferents ports, refugis i tabernes també han aportat el seu granet de sorra en fer-nos incalculablement més amable la vida del doctorant. Moltes Gràcies a en Joan, a en Carlos, Neus i Judith del PN *lab* un plaer haver iniciat aquesta aventura amb vosaltres a prop. Gràcies a tota la magnífica gent del Bigas *lab* Erika, Jessica, Carlota, Eva, Roshani, Anna, etc. per aguantar-nos sempre amb un somriure. Gràcies Marta Garrido, Àlex i Marc, tots gent del *lab* d'en Gabriel Gil, com també infinites gràcies a ell mateix per sempre que tinguérem un dubte no necessitar res en respondre'l amb diligència i entusiasme. També absoluta gratitud a na Silvia, na Laura i na Conchi per ser les veïnes tan simpàtiques i pacients que podíem tenir en una aventura així. Molt importants en el dia a dia dels mariners de l'IMIM són els tres encarregats de la cuina: Montse, Jose i Paqui. Gràcies pel vostre saber fer i la vostra feina que sempre vàrem saber vestir i farcir

quan va caldre de riures. Gràcies en Carlos per la seva simpatia única com a rei mag portant-nos regals cada dia. També creiem que es mereixen un gràcies a les noies de recursos humans per a fer-nos rasa i senzilla la paperassa vària de fer un doctorat. Finalment, enviar un moltes gràcies a tota la gent dels diferents laboratoris, sales i despatxos que quan ens veien volar pel passadís dalt d'un silenciós patinet llarg o dalt d'una cadira de rodes amb la pota inutilitzada o xiulant himnes nacionals o revertint la imatge que hem construït de científic seriós no ens feien una mirada crua i enutjada de desaprovació o llàstima sinó que tenien un somriure per a nosaltres: Lorena, Sílvia, Heleia, David, Marta, Miguel, Aïda, Ramón, Xavi, Patxi, Oscar, Joan, i un llarg etc. Gràcies a tots.

Record i agraïment a tota la gent del PRBB.

I si parlàvem del mar del doctorat també podríem fer la metàfora bonament amb qualsevol altre ambient que requereixi de temps i atenció. Així doncs, les arrels d'aquest arbre en què metaforitzem la tesi que us presentem van créixer en la terra abonada que és el fecund laboratori de *Neurophar* de la UPF. Allà la llavor de la investigació mèdica començà a brotar durant les pràctiques d'estiu notables que hi férem durant la carrera i poc a poc anar agafant soltesa i forma fins a permetre les primeres fulles emergir. Amatents, d'aquella època en guardem molts grans records i el més que sincer agraïment per a totes les persones que hi trobàrem: uns ultraistes gràcies a na Laura Cutando, Maria, Arnau, Emma, Xavi imprescindibles personatges amb qui compartirem molts i molts esdeveniments durant aquells estius. Gràcies Blanca, Aurelijus,

Sami, Andreea, Carmen, África, Ainhoa, Carmen, Cristina, Juli, Paco, Dulce, Raquel, Neus, Roberto, Isma, Xevi, Samantha, Rocío, Elk, etc. I gràcies als nous que quan l'arbre movia massa les fulles i rodolàvem a fer-los una visita no ens fèieu fora sinó que sempre ens tractàveu amb cordialitat i somriures. D'aquest part del bosc que ocupa l'arbre de *NeuroPhar* moltes gràcies especialment a tu Calvo. Tu i la teva tutela acabàreu de donar forma a la motivació que sempre es necessita per a fer un doctorat i a aprendre com fer que valgui la pena aquesta feina a voltes tan crua i punyent. Les estones ordint experiments plegats les guardem com a records molt bonics. Sobretot gràcies a tu Thomas per deixar-nos ser les teves mans com aprenents en els darrers anys de la teva tesi. Al teu costat vam llevar-nos màscares i vàrem créixer en tots els sentits de la terminologia persona. Gràcies pels moments xampurrejar francès i regant-ho tot amb cubates, gràcies per a les converses massa erudites en matins de ressaca i la teva alegria innata. Gràcies Niko, sols amb el teu caràcter afable i divertit ho posares fàcil i gràcies per fer-nos de pont ja que quan ens vam conèixer tu viatjaves en aquells temps en la tripulació del *Chromatin Team*.

Repartiment de gràcies a tota la resta de gent de la UPF.

A tots els acòlits del grup de Fisio, als d'Immuno, la gent de Bioevo, tots els de Biodes, a tots els del servei de Citometria on especialment ara al final hem passat força hores, i un llarg etcètera nostàlgic de gent que ens ha ajudat. Gràcies Xavier Marimón no sols per ser el millor del món sinó per ser un deu com a *tutorant* nostre. Tota la sort en el teu futur científic.

Obrint el focus dels arbres de la UPF per donar mercès a tots els sol·lícits treballadors de l'ALMU que sempre que tenies un dubte et treien la por d'haver perdut l'experiment perquè no veiem les anhelades cèl·lules al microscopi i ens sabien trobar la resposta legítima. A n'Albert Català per sempre poder comptar amb ell, gràcies de cor. Gràcies a tots els amics que hem fet als passadissos o ingerint canapès *destrangis* en els congressos i en general als centres que hi ha el PRBB. També dir gràcies al *lab* d'en Travis Stracker, que tant bé ens va acollir en la nostra visita a l'IRB.

En un punt intermedi entre biòlegs i externs hi trobem tots aquells nostàlgics del futbol amb qui hem compartit espectacle a la pista rectangular del marítim cada dimecres al matí! Gràcies per aquests essencials partidets de futbol sala que feien més lleugeres les setmanes. Igualment, emmarcats en el torneig de voleibol del univers PRBB, ens agradaria destacar en aquest agraïment a tots els mesells valents que van decidir-se a acompanyar-nos en generar un nou estil basat en el divertiment i l'alegria de jugar. Gràcies per obertament formar part del Futvolei a tots els que ho heu fet i ho feu cada dia possible: Max, Nino, Marc, Neus, Hima, Laura Soria, Andrea, Ali, Maria, Inyigol, Judith, Sergi, Germán, Audald, Clara, Neus Font i Laura Pascual.

Vestint la ronda de gratitud exaltada, també han contribuït en el inalterable creixement d'aquest arbre tota una colla de companys eminentment biòlegs, biòlegs de curs diferents i metges amb qui ultimarem la carrera i amb qui hem compartit un munt de moments meravellosos al llarg d'aquests anys. Gràcies Héctor per ser un dels

artífex d'aquest final de doctorat tant a nivell científic però sobretot infinitament més a nivell personal. La confiança que ens feies en petits detalls ens impulsava a ser millors en tot. Sempre podrem acudir a tu i això és molt bonic. Gràcies Jordi Xicola per sempre saber trobar temps per a fer una birra, un partidet de futbito o un senill te (que tenim pendent). Gràcies Gemma Rodri per sempre animar-nos i sense esperar-ne res a canvi. Gràcies, Agnès, per recolzar-me amb el teu caràcter entranyable, i també moltes gràcies Anna Montaner per sempre tenir un somriure a mode de destral per nafrar de mort els mal rollos. Gràcies Juna, Júlia, Abel, Mariona, Marina, Silvia, Xian, etc etc etc... per tot, per tant, per sempre. Ompliu cada un dels moments plegats amb un glop de d'aire fresc, literalment un alè de felicitat, com un punt de suport tant franc i talment ho feu d'una manera tant natural que a voltes sembla que sigui impossible. Mil milions de gràcies a la gent que treballa dia a dia amb decisió per aconseguir que Palestina esdevinguí un territori imbuït de pau i lliure de tota ocupació. A tots els que hi, amb esforç, en algun moment i heu contribuït amb Nos: Ulises, Adriano, Anna Sol, Aina, Marina, Ana, Linus, etc. Moltes gràcies. Gràcies especialment Laura Rodri i Roger "*Fingals*" Vinyals, ambdós no necessitareu ni dos dies per abraçar-me com a amic quasi sense saber d'on venia i sempre em sento en família amb vosaltres.

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XIX51419 4212205 1415 209143 1611812112519 16518 524161851919118 1721114 651293 513 195142015 4'8122518 2018152120 2114 7182116 31513 512 17215 6151813513. I132 12519 1415192018519 31519519 1651815 1951316185 1853151226120 16518 20152019 221519122018519. XIX152145 12519 16151721519 31519519 17215 1415 513 3114191185 13199 4'51920118 151772112121519. IX, 31513 4591 512 16155201, 195 17215 51431181 17215 1415 2119 1453519199209 2119 209143.

      Cada un dels que m'heu tret el mono futbolístic.

I que coneixeu cap arbre que pugui créixer sense una estrella que li aboqui llum a dojo, o a cap mariner autèntic sobreviure sota un cel recelós d'estrelles? En la lògica de la metàfora que hem plantejat les estrelles són els moments gaudint del món del futbol. En som uns malalts apassionats i cada cop que podíem xutar una pilota xutàvem de cop els mal-de-caps que ens han anat assaltant durant i per aquesta tesi. Són moltes les persones amb qui convius i tan límpidament com pugui els resumiria en tres grans grups d'amics gegants.

- Un primer gràcies a cada un dels jugadors que, al costat de Nos, en algun moment han format part del senzill però simpàtic *Camprodon Team*. Poder jugar amb vosaltres una mica un vespre per setmana feia molt més fàcil assolir amb empenta el dia a dia del *lab*. Gràcies a tots els que hi vam ser. Gràcies a en Pupi, Marc G., Pep, Oleguer, Marc O.,

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- Als diferents seleccionats que algun cop hem conviscut en les files de la selecció de la UPF de la mà de FernanSacu. Tots, tant els dels darrers anys: Xonfry, Segu, Rove, Andreu, Rocky, Nil, Willy, Pope, Pablo, Vicentet, Xavi, Campa, Erick, Nacho, Joan, etc. i als mítics dels anys precedent que són: Uri Roig, Ricki, Joaco Liso, Ramón Vilaplana, Marcel, Uri. Ll., Bolado Segarra, Raure, Monti, etc. amb especial nostàlgia gràcies al que en fou míster i motor durant tants anys en Josep Ma Roma, i els *capis* en Max Carbonell i el màgic Jordi *Xory* Juncà, la bella guàrdia. Al vostre costat he intentat gaudir del futbol des de la seva vessant més competitiva que només es viu en fases eliminatòries com aquelles en què vam participar.

- Per acabar, gratitud infinita a un equip que ens ha donat estima sense esperar res més a canvi que seguíssim volent recórrer el camí junts. El Verges F.C. Moltes gràcies per les oportunitats a totes les persones que durant 10 anys ens han mimat perquè poguéssim jugar a futbol allà en un ambient agradable i unes condicions excel·lents, àdhuc amb el que implica entrenar només un dia a la setmana i gaudint del que omple una autèntica família futbolística. Gràcies a tots. Quin baül de records, des del primer dia que en formem part quan l'Àngel Roig ens hi portà fins a l'actual capità d'aquest increïble equip, n'Adrià Sabater. Moltes gràcies de tot cor.

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De Nos a tots els que heu aguantat el biòleg que portàvem dins.

Així doncs, mentre fèiem créixer l'arbre en el mar del doctorat regalant-li llums de futbol en la justa mesura descuidàvem, esmarrits, moltes coses i per sort això només s'ho pot permetre qui mesura una estructura i uns fonaments increïblement ferms i segurs. En aquesta metàfora que convertim ara la tesi, res hauria estat literalment possible sense la complicitat d'un seguit de persones. Què dir de la colla d'amics de Girona que mereixen molt més que un breu esment en aquesta tesi amb qui coincidim menys del que ens agradaria. Nos, en Joan Pau, esperem que seguiu sent tant honestos com heu estat sempre amb nosaltres i seguiu volent-nos esperar i tenir a prop com a part de les vostres vides. Nos, ho farem mentre ens deixeu poder-ho fer. A veure si ara que hem acabat la vilipendiosa, a voltes, etapa recuperem el temps perdut amb escreix. I doncs, amb la seguretat de deixar-nos algun nom, restem contents sabent que us donem les gràcies que tant us mereixeu: Gràcies de cor a en Jaume R^2, Marc Cassany, Rafel i Cristina, Elisabeth, Roser, Uri Serra, Vallès, Pere i Cristina, Oriol *Fafa*, Jordi Miquel, Jordi Totti Bayer, *Fafa* i Blanca, Mònica, Jordi *Ferna*, Ignasi, Quim, Anna i Unai, Edu, Jaume FP, etc.

Queda palès en molts moments que per aixecar aquesta casa són unívocament definitius els diferents estius passats al Collell i especialment els darrers anys on sempre hem comptat amb el vostre suport i comprensió. Gràcies Jordi i Meritxell per la vostra estima. Trobareu sempre la nostre gratitud a tota la vostra família i també a tots els qui treballen amb vosaltres, que sempre ens fan sentir una

enèsima part d'aquesta gran família que fa tant bona feina entre les muntanyes del Pla de l'Estany i la Garrotxa. Per a tots vosaltres un petit record per a haver-vos tingut tant abandonats els darrers semestres. D'aquí també en sorgí una amistat en què hi confiem ullclucs amb n'Ana Quintano. Moltes gràcies per les visites curtes sí però boniques i perquè les seguim podent fer en el futur! Un petó espaterrant per a tu, per a en Rakan i pel vostre *retoño*.

Sense cap mena de dubte, molta gent del col·legi Bell-lloc del Pla també mereix un esment en aquests agraïments, a part dels ja anomenats, ja sigui perquè ens van animar a agafar aquest camí radicalment com en Manel Montoliu com perquè quan hi hem tornat ens han animat a seguir, malgrat les dificultats, en aquesta vida aclapara del doctorant. La llista és llarga així que només faré un genuí esment a la parella formada per en Pepe Muñoz i na Mariona responsables d'insuflar-me l'energia necessària amb els seus bons advertiments i arribant on jo no sempre puc. Una muntanya amb infinitat de mercès per a tothom.

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I ben contents hi posem un punt i final. Que sigueu tots molt feliços.

Nos, en Joan Pau Cebrià i Costa

Abstract

Oxidation of H3 on lysine 4 (H3K4ox) by lysyl oxidase–like 2 (LOXL2) generates a new H3 modification with an unknown physiological function. We determined from cell lines and patient–derived xenographs (PDXs) that the triple-negative breast cancer (TNBC) subtype has higher levels of LOXL2 and H3K4ox, and a more compact chromatin, than other breast cancer subtypes. H3K4ox is mainly in heterochromatin, where it controls compaction and inhibits the DNA damage response (DDR). Knocking-down LOXL2 reduced H3K4ox levels and "opened" chromatin, resulted in DDR activation and increased susceptibility to cell death. This critical role of oxidized H3 in chromatin compaction and the DDR suggests that targeting it to force open chromatin could be a way to sensitize TNBC cells to conventional oncological therapy.

Resum

L'oxidació de la histona 3 en la lisina 4 (H3K4ox) per acció de la lysil oxidase-like 2 (LOXL2) genera una nova modificació de la H3 amb una funció desconeguda. En aquesta tesi, hem determinat tant en línies cel·lulars com en xenografs derivats de pacients (PDXs) que el subtipus de càncers de mama triple negatiu (TNBC) presenta uns nivells elevats de LOXL2 i H3K4ox, i una cromatina més compactada que els altres subtipus de càncer de mama. L'H3K4ox es troba majoritàriament en l'heterocromatina, on controla la compactació de l'ADN i inhibeix la resposta al dany en l'ADN (DDR). Eliminar la LOXL2 redueix els nivells d'H3K4ox i "obre" la cromatina, provocant l'activació del DDR i fent que augmenti la susceptibilitat a la mort cel·lular. Aquest paper crucial de l'oxidació de l'H3 en la compactació de la cromatina i el DDR suggereix que forçar l'obertura de la cromatina podria ser una manera de sensibilitzar les cèl·lules TNBC a teràpies oncològiques convencionals.

Index

Abbreviations

A: Adenine

ATAC: Assay for Transposase-Accessible Chromatin

ATM: Ataxia Telangiectasia Mutated protein

ATR: Ataxia Telangiectasia and Rad3-related protein

Bp: Base pair

C: Cytosine

ChIP: Chromatin ImmunoPrecipitation

Chk1: Checkpoint kinase 1

Chk2: Checkpoint kinase 2

CSC: Cancer Stem Cell

CTs: Chromosome Territories

DDR: DNA Damage Response

DNA: Desoxyribonucleic Acid

EC: Euchromatin

EMT: Epithelial-to-Mesenchymal Transition

ESC: Embryonic Stem Cell

G: Guanine

H1: Histone 1

H3: Histone 3

HC: Heterochromatin

HP1: Heterochromatin Protein 1

HR: Homologous Recombination pathway

IF: Immunofluorescence

IR: Ionizing Radiation

LADs: Lamina-Associated Domains

LOXL2: Lysil Oxidase-Like 2

LTQ domain: Lysine Tyrosilquinone domain

MET: Mesenchymal-to-Epithelial transition

mRNA: messenger Ribonucleic Acid

NHEJ: Non-Homologous End-Joining pathway

PDX: Patient-Derived Xenograph

PR: Progesterone Receptor

PTM: Post-Translational Modification

RA: Retionic Acid

RNA: Ribonucleic Acid

RNAPolII: RNA Polymerase II

ROS: Reactive Oxygen Species

SNAI1: Snail family transcriptional factor 1

T: Thymine

TADs: Topologically Associating Domains

TGF-β: Transforming Growth Factor beta

TNBC: Triple-Negative Breast Cancer

1. INTRODUCTION

In the following sections, I will describe the different molecular bases that regulate the biology of a cell from a chromatin perspective (section 1.1), the field of cancer in general, and more specific in the topics which the work I hereby present belongs (section 1.2), and finally the lysil oxidase-like 2 enzyme (LOXL2) which activity is under study in the lab currently (section 1.3).

1.1. A CHROMATIN PERSPECTIVE

1.1.1. The basic structures

a) **Nucleosomes**

DNA (desoxyribonucleic acid) contains all the information necessary to generate and maintain a living organism (Thomas and Kornberg 1975). It is made out of two polymeric strains of 4 different nucleotides (bases): Adenine (A), thymine (T), guanine (G) and cytosine (C). DNA forms an antiparallel double helix bound together with hydrogen bonds between the bases forming a double-stranded chain (Watson and Crick 1953) (Fig.i1). Together with and a group of proteins called histones, DNA is compacted into the nucleosomes (Luger et al. 1997), the basic element of chromatin inside the nucleus (Hewish and Burgoyne 1973; Kornberg 1974).

Figure.i1. Photograph of a rough scale model of the structure of DNA. An original model from Watson & Crick published in 1953. The two antiparallel strains forming a double helix and maintained together by hydrogen bonds between the bases (Crick and Watson 1954).

In eukaryotic cells, nucleosomes consist of 147 base pairs (bp) of DNA wrapped around two tetramers of histones (Kornberg 1974). These two tetramers form the core of the nucleosome. Each tetramer is formed by HISTONE 2A (H2A), HISTONE 2B (H2B), HISTONE 3 (H3) and HISTONE (H4) (Fig.i2). In addition, there are different variants for each histone protein with different distribution along the genome and specific features (Weber and

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Henikoff 2014). Protruding from the core of the nucleosome, there are particular domains called the tails of the histones (Strahl and Allis 2000; Jenuwein and Allis 2001). The tail of a histone is the N-terminal region of each histone, and its structure and post-translational modifications (PTMs) are of crucial importance in nucleosome biology.
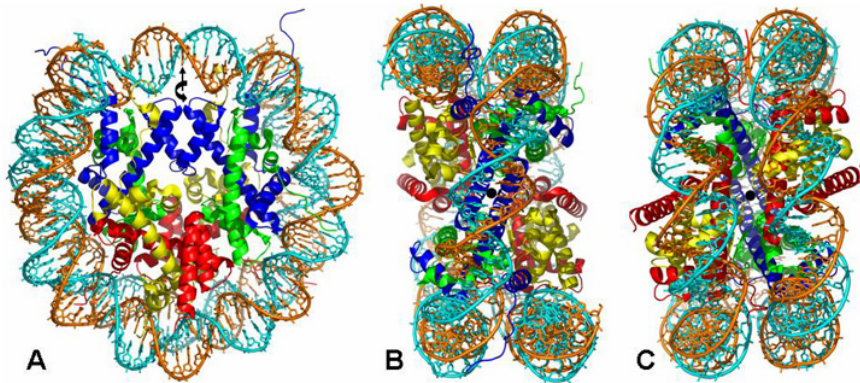

Figure.i2. Crystal structure of the core of a nucleosome. Classic image of the DNA double helix (orange and celeste) wrapping twice the histone core composed by two tetramers of histones H2A (yellow), H2B (red), H3 (blue), H4 (green). (A) Top view of the core of a nucleosome along the DNA superhelical axis. (B) and (C) Side views of the nucleosome perpendicular to the DNA superhelical axis.

A region of 20-90 bp of free DNA called linker DNA separates nucleosomes (Bednar et al. 1998). The length, accessibility, and the structure of the linker DNA determine also the role of the surrounding DNA. In addition, HISTONE 1 (H1) protein binds to the linker DNA adding one step of compaction to the chromatin (Woodcock, Skoultchi, and Fan 2006). The presence of H1 is linked with the degree of accessibility between the nucleosome-entering

and exiting DNA helices and further of all the chromatin compaction (Sivolob and Prunell 2003; Kepper et al. 2008) (Fig.i3).
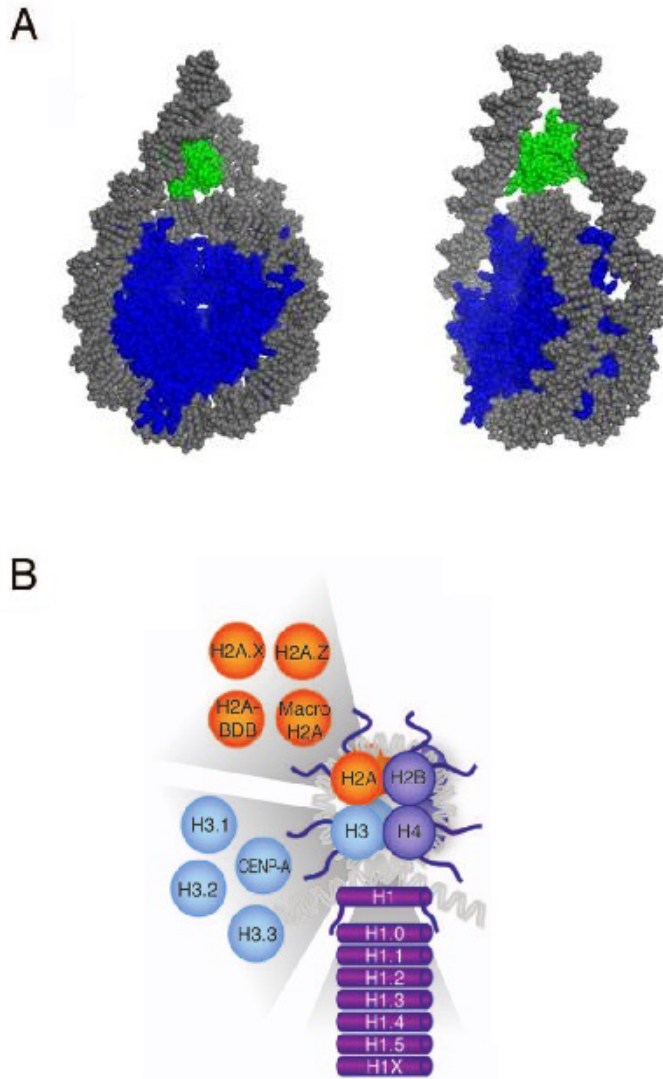

Figure.i3. Nucleosome representation with the linker Histone 1. (A) Remarkably, histone 1 (green) couples between the double helix of DNA (grey) that is entering and exiting from the nucleosome (blue) (Cutter and Hayes 2016). (B) Schematic cartoon of the nucleosome with the linker H1 and different examples of histone variants (Tollervey and Lunyak 2012).

Based on the DNA sequence, chromatin can be divided into different regions (Ogbourne and Antalis 1998). The most important elements are the genes. Although modern science does not offer a precise definition of a gene, gene consists of a region of DNA that contains the information necessary to produce a specific protein (Pearson 2006). Genes are composed of exons, regions that will be codified into protein, and introns, regions that will not be part of the protein. Selected genes will be transcribed into messenger RNAs (mRNAs), and finally translated into proteins (Watson and Crick 1953). Regions that do not code for a protein are important in the regulation of the coding parts of the genome. In last years, non-coding DNA (ncDNA) elements reached increasing importance as regulatory elements of both gene regulation and cellular fate (van Bakel et al. 2010; Djebali et al. 2012; Consortium 2013). Different functions arose in the last years for ncDNA elements, from non-coding functional RNAs (ncRNA) (van Bakel et al. 2010) to protection of the genome (Qiu 2015).

Important for gene regulation are promoter and enhancer elements (Andersson 2015). Promoters are elements of open chromatin upstream of the transcriptional start site of the gene (TSS). Transcription factors (TFs) bind promoters specifically and promote or repress gene transcription by either recruitment of RNA polymerase II (RNAPII), or recruitment of other repression regulators. Enhancers elements are usually several kilobases away from the transcriptional start site (Ogbourne and Antalis 1998; Plank and Dean 2014). They are called enhancers when promote gene expression, insulators when work indirectly to other elements

and silencers when repress gene expression (Wallace and Felsenfeld 2007). The importance of enhancers and silencers rely on the folding capacity of chromatin that allows the gathering sequence-remote (cis) regions of the DNA (Cremer and Cremer 2010) (Fig.i4).
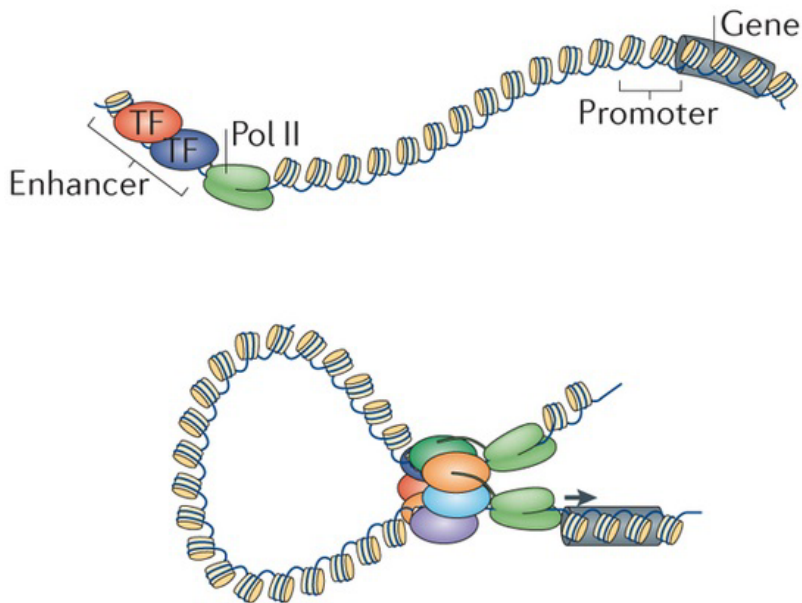
Figure.i4. Gene transcription requires direct contact between enhancers and promoters. The direct contact between the promoter and its enhancer occurs throughout the looping of the chromatin fiber and allowing the transcription of the gene by the RNAPolII (green). Several transcriptions factors are involved in this process (Pombo and Dillon 2015).

In addition, other important mechanisms above the regulator elements based on the DNA sequence control the gene expression. These mechanisms are known as epigenetics. In the early 90s, epigenetics was described as anything other than DNA sequence

that influences the development of an organism (R. Holliday 1990). Hence, any modification on the chromatin without altering the DNA sequence is considered as epigenetic modification. Here, I will point out two modifications: DNA methylation and histone post-translational modifications.

DNA methylation consists in the addition of a methyl group to the DNA molecule (Schübeler 2015). Both cytosine and adenine bases are suitable to be methylated. It has been firstly described as a defense from bacteriophages. Nowadays, they are described to be a silencing mark reducing the transcriptional activity of chromatin. Adenine methylation (N^6-mA) is almost non-present in mammals, and was thought to be exclusive of bacteria and plants, however recent evidences suggest important roles in eukaryotic organisms. Controversially, a model where this mark is promoting active transcription has been described in insects, nematodes and a green algae (G. Zhang et al. 2015; Greer et al. 2015; Ye Fu et al. 2015). Nevertheless, in mammalian embryonic cells, N^6-mA has been described a newly silencing element for transposon element (T. P. Wu et al. 2016).

Cytosine methylation is largely studied and nearly all of them localize in cytosine residues of CpG dinucleotides, what is referred as CpG islands. Most of these CpG islands localize close to the 5' region of genes where they repress transcription (Lister et al. 2009). DNA methylation is carried out by DNA methyltransferases enzymes (DNMTs) and it can be removed by the TET family of enzymes through an oxidation reaction (Yi Zhang and Kohil 2014).

b) **Chromosomes**

Chromatin is physically divided into structures called chromosomes (Cremer and Cremer 2010). In human cells, each cell contains 44 autosome chromosomes (1-22) and 2 sex chromosomes (X and/or Y) (Tjio and Levan 1956; Ford and Hamerton 1956). Half of them belong to each one of the progenitors. Chromosomes can be observed in the metaphase of the cell cycle as an X-shaped structures of two chromatids containing the two copies of information after cell replication (Adolph 1980; Belmont et al. 1989). In the metaphase, chromosomes have three well-characterized regions: the centromere where the two sister chromatids are joined (Pluta et al. 1995); the telomeres at the end of the chromosomes (Olovnikov 1973; Blackburn, Epel, and Lin 2015); and the chromosome arms (Fig.i5). In the interphase, chromosomes are decondensed and distributed over the nucleus (Cremer and Cremer 2010).
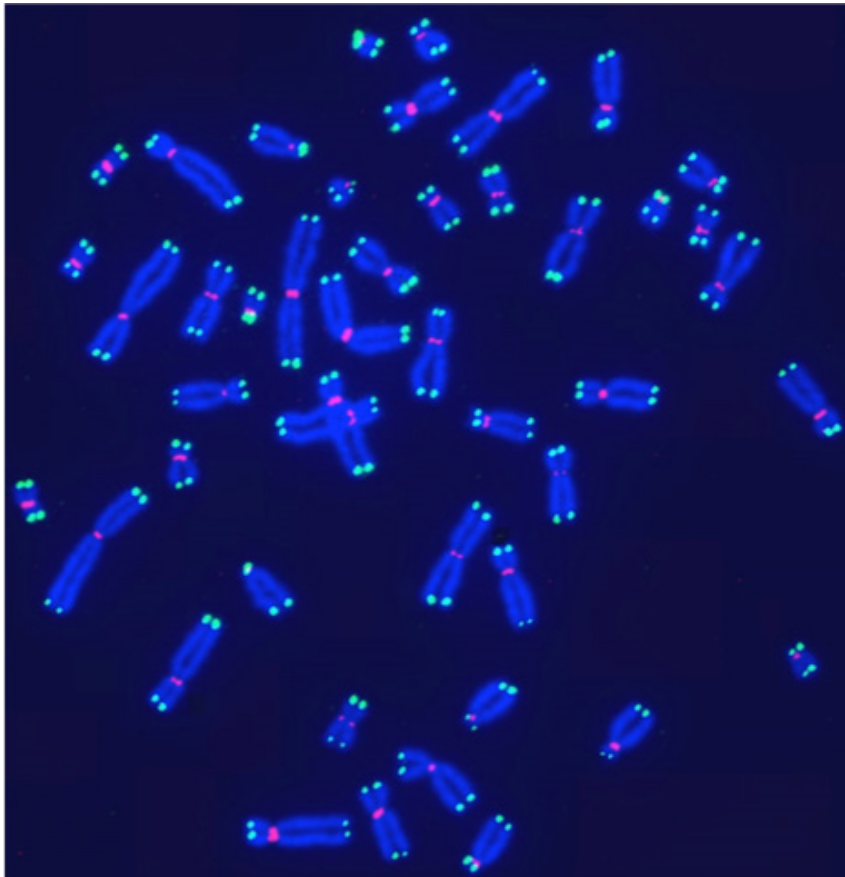
Figure.i5. Human metaphase chromosomes observed using digital fluorescence microscopy. Metaphase chromosome spreads in a quantitative fluorescence in situ hybridization (Q-FISH). Chromosomes are marked using DAPI (blue). Different probes were used to target telomeres (green) and centromeres (red) (from Shay/Wright lab).

1.1.2. A nuclear perspective

a) **Euchromatin vs. Heterochromatin**

Traditionally, interphase staining of the chromatin distinguishes between two main regions: Euchromatin (EC) and Heterochromatin (HC) (Fig.i6). HC presents a more condensed structure than EC (Brown 1966; Becker, Nicetto, and Zaret 2016). Further

experiments elucidated more difference between both regions: on one hand, HC is characterized by being less accessible or closed, enriched in repetitive sequences, present in the centromeres and telomeres of chromosomes and is replicated in late S-phase stages. On the other hand, EC is less condensed and more accessible or open, hence, easily transcribed, gene enriched, present in the chromosome arms and is the first region to be replicated during the S-phase (Bo Wen et al. 2012; Feng and Michaels 2015; Tamaru 2010).
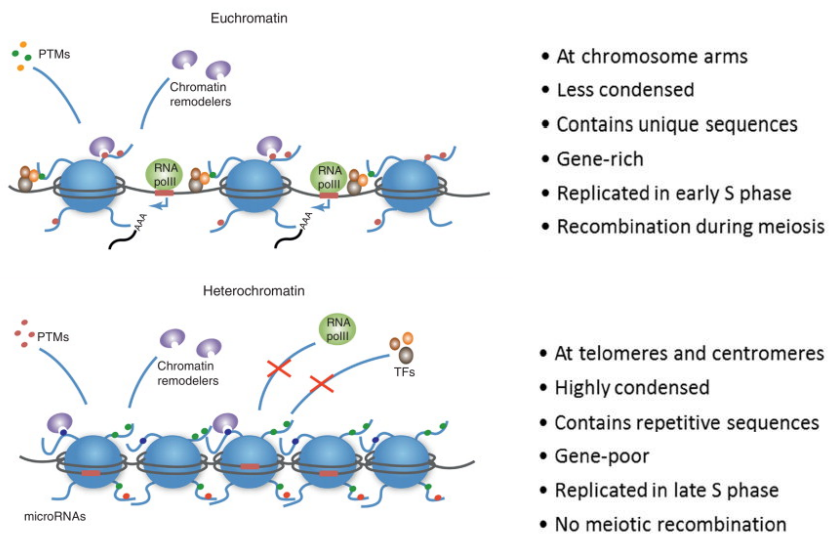
Figure.i6. Properties of euchromatin and heterochromatin. Brief list with the main properties of euchromatin and heterochromatin. However, some exceptions can arise from the two groups (Adapted from (Grewal and Elgin 2007; Croken, Nardelli, and Kim 2012)).

b) **Chromosome Territories and the High-throughput data**

Besides this, more evidence in the last years suggested a high order of complexity in the architecture of the chromatin inside the nucleus: the Chromosome Territories (CTs) (Fig.i7). The CTs

consist of specific regions inside the nucleus for each chromosome (Cremer and Cremer 2010; Stevens et al. 2017). Laser UV-microirradiation experiments confirmed, already in the 80's, the presence of these CT in the nuclei of diploid Chinese hamster cells.
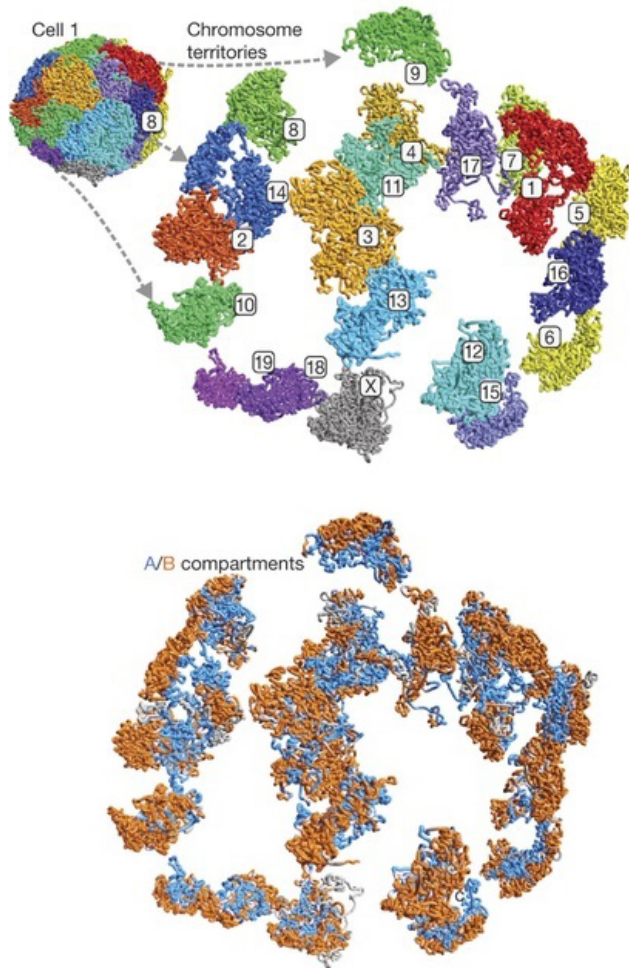
Figure.i7. Chromosome Territories in the interphase nucleus. High-throughput data from single-cell Hi-C experiments allow, recently, a better modeling of the chromatin architecture inside the nucleus. 3D structure of an haploid mouse ES genome with expanded view of each chromosome (left) and the distribution of the compartments A (blue) and B (red) (right) (Stevens et al. 2017)).

Moreover, in the last decades, high-throughput sequencing technologies allowed a better understanding of CTs and added new concepts in the organization of the chromatin inside the nucleus. Chromatin immuno-precipitation sequencing (ChIP-seq) of different chromatin-related proteins addresses different regions of the chromatin based on their specific characteristics (van Steensel 2011). For example, the colourful division of the chromatin in drosophila cells can discriminate five different types of chromatin based on ChIP-seq experiments of 53 chromatin proteins (Filion et al. 2010) (Fig.i8).
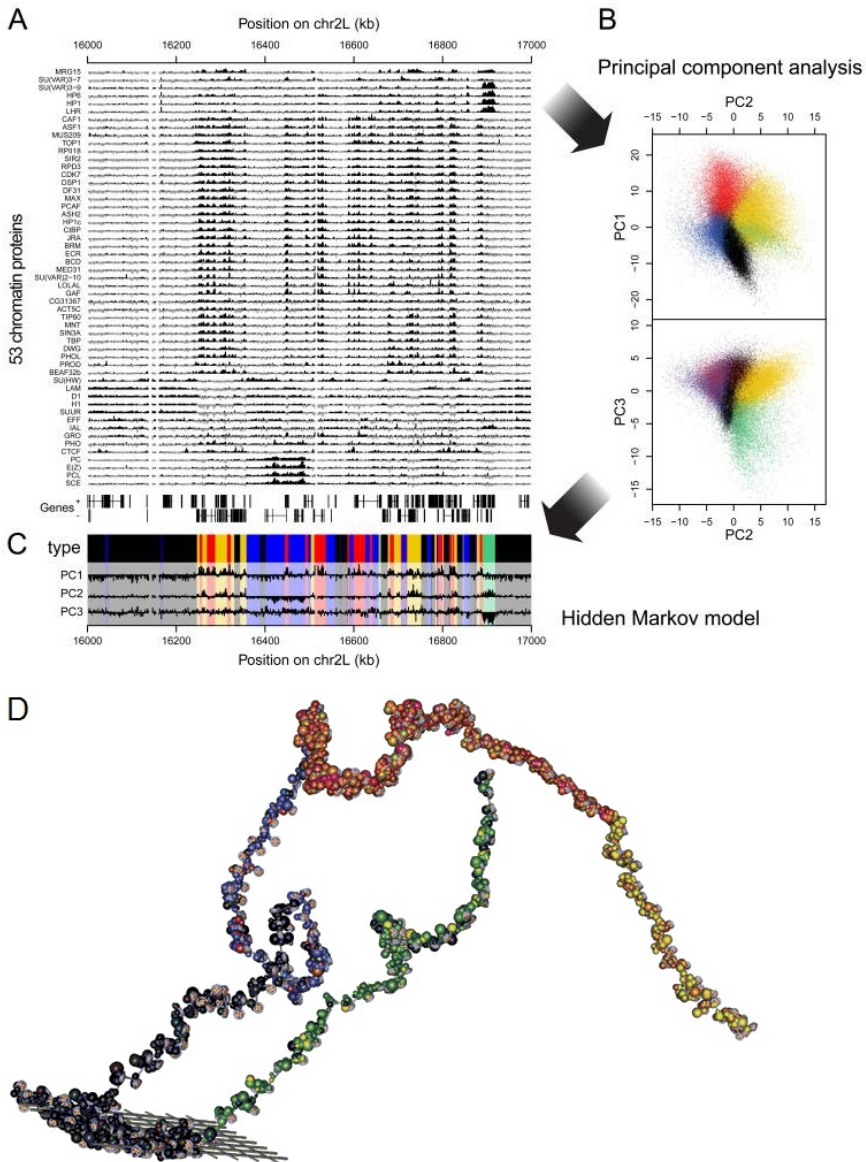
Figure.i8. The colors of the genome. (A)(B)(C)After systematic integration of 53 protein location maps in the embryonic Drosophila melanogaster cell line Kc167 chromatin can be divided into 5 big compartments. (D) Cartoon representation of the 5 compartments: Black, used to be associated to the nuclear lamina; Blue, mostly repressed by the Polycomb group (PcG) of proteins containing genes involved in the regulation of the developmental process. Red and yellow, being these ones the highly transcribed ones, with the red showing an enrichment of cell-specific genes and yellow the more ubiquitous ones. Green,

containing the heterochromatin protein 1 (HP1) related genes (Filion et al. 2010; van Steensel 2011).

In fact, genomic architecture can clearly distinguish between two compartments A and B that always cluster together and segregate from each other (Fig.i7). Moreover, A compartment is the more accessible one where mostly all high expressed genes lie, and B the more compacted within the nucleolus and close to the nuclear lamina (Stevens et al. 2017). Similarly, accessibility studies have highlighted important characteristics of nuclear architecture. All of them discriminate chromatin by its capacity of being accessible (Fig.i9).

- DNAse-seq and MNase-seq techniques are based on the ability of different nucleases to cut the free DNA. Therefore, DNAse-seq shows the accessible regions of the genome because of the activity of DNAse I on free DNA. In addition, deep sequencing allows to study the foot-printing of proteins on DNA (Boyle et al. 2008).

- MNase-seq elucidates the positions of the nucleosomes in the chromatin by a complete digestion of the free regions of DNA by MNase, a single-strand-specific endo-exonuclease, activity (Rizzo, Bard, and Buck 2012).

- Faire-seq gives information about the accessible regions of the genome. It is based on the different solubility of free DNA or DNA cross-linked with proteins using phenol-chloroform separation (Giresi et al. 2007).

- ATAC-seq allows inferring accessible regions of the genome by the activity of Tn5 transposase. This transposase fragments DNA are tagged with adapters followed by library preparation. Hence, ATAC-seq allows to uncover open chromatin, nucleosome positioning and protein footprints genome-wide (Buenrostro, Wu, Chang, et al. 2015).

- Finally, the chromosome conformation capture techniques (3C, 4C, 5C and Hi-C) (Fig.i10) are used to describe the interaction between DNA regions (Sati and Cavalli 2016; Schmitt, Hu, and Ren 2016; Denker and de Laat 2016). These techniques report on the relative frequency in the cell population by which two loci are in close spatial proximity. After cross-linking step, DNA is cut by enzyme digestion into small fragments and ligated again to generate unique hybrid DNA molecules.
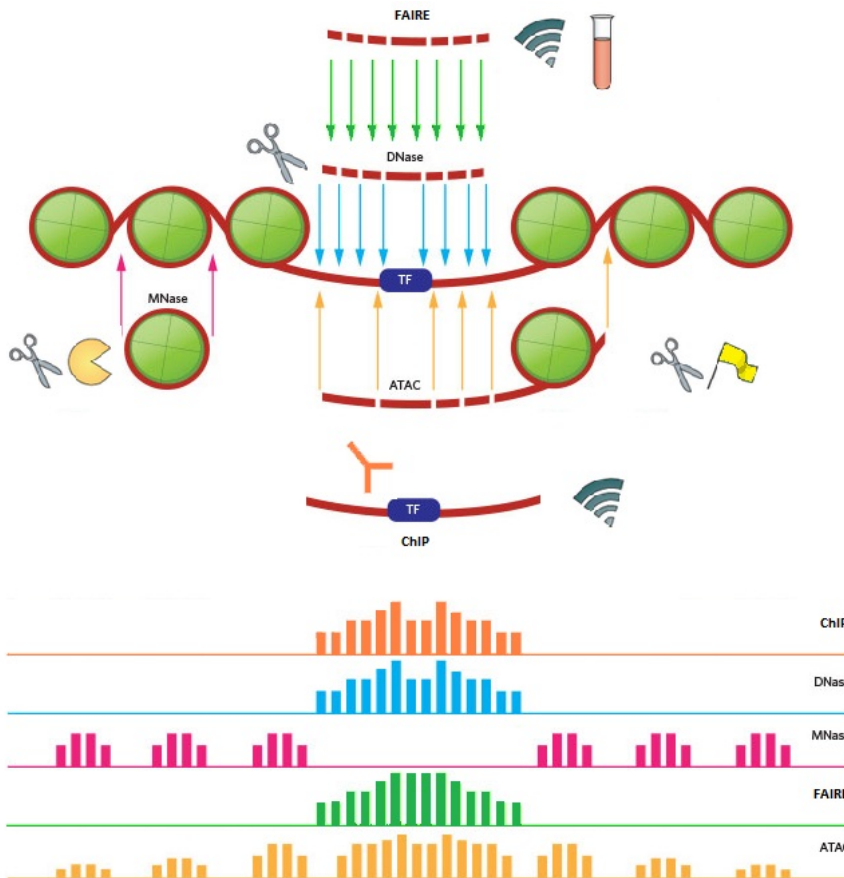
Figure.i9. The accessibility studies. Simplified cartoons of the different techniques to infer the chromatin distribution and foot-printing of transcription factors (TF), nucleosome position (green circles) and chromatin accessibility. ChIP-seq (orange) fragments the chromatin by sonication and enriched the chromatin region using an antibody against the studied protein; DNase (blue) uses an enzyme to digest the chromatin in the accessible regions; MNase (pink) uses a an enzyme that digest the free regions of the genome arising the chromatin in nucleosomes; FAIRE-seq (green) fragments the chromatin using sonication and separated the free regions of DNA by phenol-chloroform separation; ATAC-seq (yellow) uses a transposase to digest the chromatin and tagged it. (Adapted from (Tsompana and Buck 2014a; Meyer and Liu 2014).

Using the 5C and Hi-C techniques, new organization steps were defined (Dixon et al. 2012; Czapiewski, Robson, and Schirmer 2016). Chromosomes are composed of discrete topologically

associating domains (TADs). These TADs are separated from each other by striking borders. Interestingly, these borders correlate with CCCTC-binding factor protein (CTCF) positions on the genome (Holwerda and de Laat 2013; Lupiáñez et al. 2015; Gómez-Marín et al. 2015). TADs are proposed as the fundamental structural units of the genome. These TADs confined most of the interactions inside each TAD and little of them between them. TADs' contacts comprise the interaction between enhancer and promoters regions and genetic coregulation is observed inside each TAD. TADs are maintained between cells, tissues and species suggesting an important role of these discrete contacts in the chromatin organization (Denker and de Laat 2016; Dixon et al. 2012; Pombo and Dillon 2015; Nora et al. 2012).
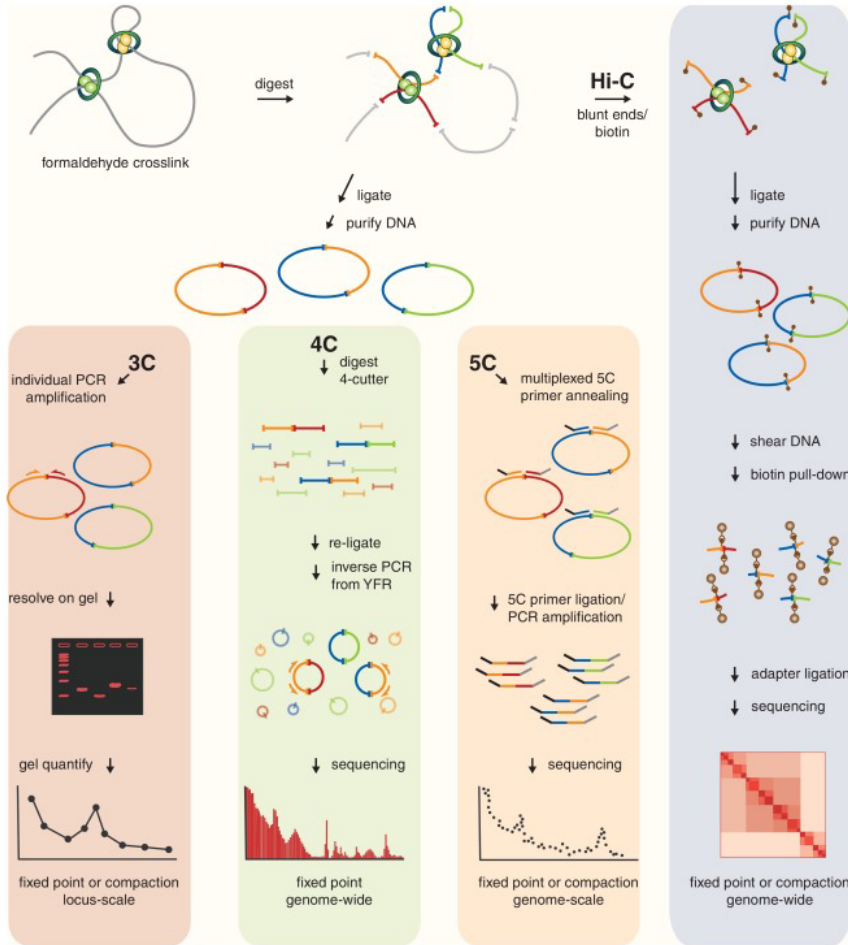
Figure.i10. The chromosome conformation captures (3C). First, chromatin is crosslinked with formaldehyde in order to capture the chromatin organization. Then, a restriction enzyme digests the chromatin generating DNA fragments. In 3C, 4C, 5C these fragments simply re-ligated into circular products. For Hi-C, before the ligation, the resection fragments are marked using biotinylated nucleotides. During 3C, in order to measure the frequency of contacts, primers are designed upstream the specific cut site. Then, a PCR is used to amplify the contacts and these are quantified on agarose gel. For 4C, a secondary digestion using a more frequent cutting enzyme is done on the 3C library. Then, fragments are ligated and circularized. Since, this technique will arise all the contacts against the specific region of interest where the primers are designed. In 5C, the primers are designed against the restriction ends. After primer annealing they are ligated and prepared in a multiplex settings to quantify the abundance of contacts between different regions. Finally, in Hi-C, the prepared libraries are sonicated and enriched using streptavidin beads. Then, the fragments are ligated to adaptor oligos and sequenced (X. Q. D. Wang and Dostie 2017)

c) **Nuclear structures**

As explained, chromatin resides within the nucleus. The nucleus envelope is formed by three different structures: The nuclear membranes and the nuclear lamina (Hetzer 2010) (Fig.i11). The membranes consist in a two bilayers membrane of lipids (the inner and the outer) fused to the endoplasmic reticulum (ER). Different proteins are anchored to these membranes and remarkably a group of proteins form channels on it, the nucleoporines. The nucleoporines are big complexes of proteins that form pores allowing the pass of proteins, RNA and ions across the nuclear membrane. Inside these two membranes and in contact with chromatin is the nuclear lamina (Czapiewski, Robson, and Schirmer 2016). The nuclear lamina consists in a mesh of fibrilliar network of intermediate filaments and membrane associated proteins. These intermediate filaments can be divided into two main groups: A-type lamins (lamins A/C) and B-type lamins (lamins B1 and B2). The different amount of one type or the other in the nuclear lamina shows different stiffness of the nucleus. Since, the presence of A-type laminas is associated to a more stiff nucleus (Swift et al. 2013) and to be particularly enriched in differentiated cells (Constantinescu et al. 2006). Nevertheless, B-type lamins has been associated with deficiencies in the morphological changes associated to migration (Tran, Zheng, and Zheng 2016) and chromatin stability (Butin-Israeli et al. 2015). The membrane associated group of proteins is composed by 60 different types of proteins (Schirmer et al. 2003). They are transmembrane proteins anchored into the inner membrane of the nucleus and mainly all of

them are able to bind lamins. Moreover some other are able to bind also to other chromatin proteins like lamin B receptor that interacts with heterochromatin protein 1 (HP1) (Q. Ye and Worman 1996). The firstly described function of the nuclear lamina was to serve as scaffold for the membranes, although in the last years new functions associated to this structure (Schreiber and Kennedy 2013). The nuclear lamina has been described to interact with chromatin tethering big regions of the genome close to the nuclear membrane, the lamina-associated domains (LADs) (Guelen et al. 2008). Moreover, this tethering is associated with a repressed state of the chromatin and indeed prevalence for heterochromatic domains is described to be proximal to the nuclear membrane. Besides, LADs are suggested to be constant between cells types, a small fraction of these LADs switch between each cell allowing to differentiate between the constitutive LADs (cLADs) and the facultative LADs (fLADs) (Peric-Hupkes et al. 2010). Alterations in any of the components of the nuclear lamina are associated with extremely threatening diseases like progeria (Mounkes et al. 2003).
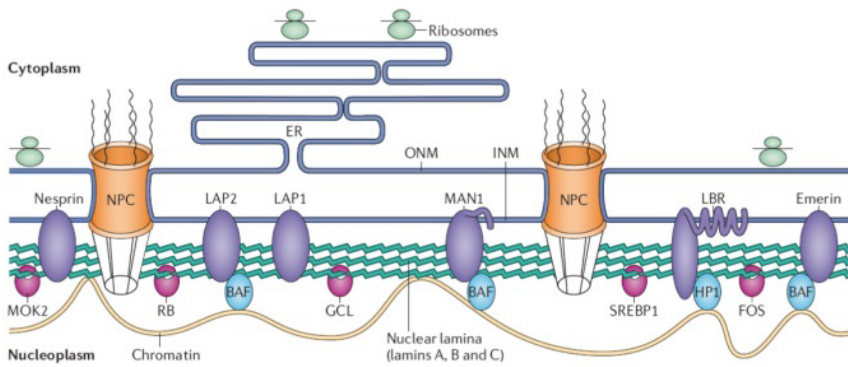
Figure.i11. The nuclear envelope. Archetypical representation of the two nuclear membranes and the nuclear lamina and of different membrane associated proteins (Coutinho et al. 2009).

1.1.3. EPIGENETICS ON THE HISTONE TAIL

a) **Epigenetics marks on histones**

As explained, a particular domain of histones protrudes from the nucleosomes: the histone tail domain (Jenuwein and Allis 2001; Strahl and Allis 2000). This domain establishes a new epigenetic step of regulation. Hence, the tail domain becomes an exquisite platform for variations in the regulation due to different covalent post-translational modifications (PTMs).

There are more than 50 described PTMs in different residues of the histone tail domain of the nucleosome (Kouzarides 2007; Lawrence, Daujat, and Schneider 2016) (Fig.i12). The ones more characterized are acetylations, methylations and phosphorylations. However, several others have been described as ubiquitinations, sumoylations, etc. A new modification described in our group is the deamination

of lysine 4 trimethylated in histone 3 (H3K4me3) by the catalytic activity of the lysil oxidase like protein 2 (LOXL2) (Herranz et al. 2016).
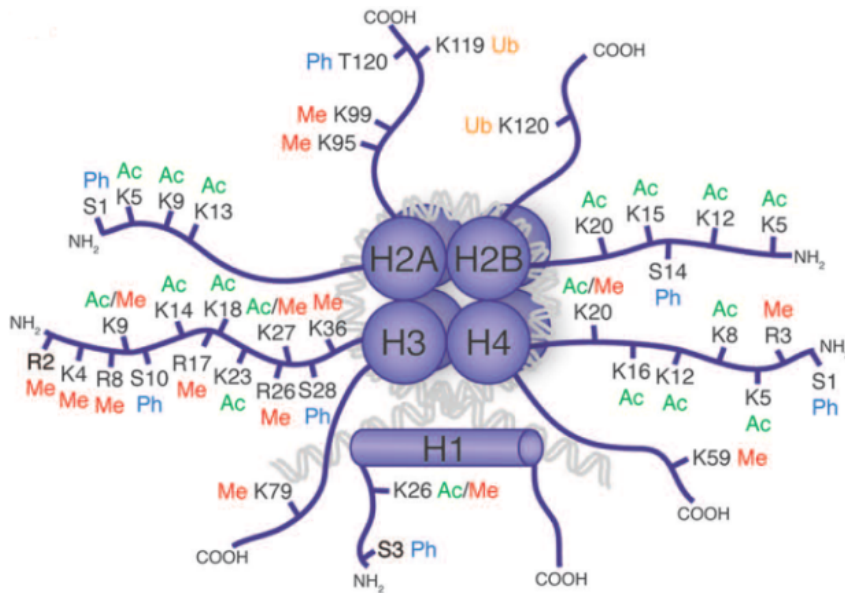
Figure.i12. Epigenetic marks on the histone tail. Classic image of a nucleosome with the linker H1 and with some of the different PTMs in the histone tail that have been described. Acetylation (Ac); Methylation (Me); Phosphorylation (Ph); Ubiquitination (Ub). (Cota, Shafa, and E. 2013).

Modifying the histone tail domain could be sufficient to alter the nucleosome structure and lead to a change of the regulation as a consequence of a direct structural perturbation. For example, lysine 16 acetylation in histone 4 (H4K1ac) disrupts the electrostatic interaction between histones (basic molecules) and DNA (acid molecules) (Bradley et al. 2006; Pepenella, Murphy, and Hayes 2014). Hence, this less compacted chromatin structure promotes chromatin accessibility (Bell et al. 2010). Moreover, modifying the

histone tail domain may generate new targets recognizable by specific proteins. Trimethylation of lysine 9 in histone 3 (H3K9me3) is one example as it is recognized by Heterochromatin protein 1 (HP1) as a signal to compact the surrounding genomic region (Lehnertz et al. 2003). Furthermore, post-translational modifications in the histone tail are dynamic and, for example, suffer change during the cell cycle (Santos-Rosa and Caldas 2005). Indeed, the distribution of these epigenetic marks causes different and particular effects on those genomic regions where is distributed (Fig.i13). In short, the different histone PTMs on the histone tail correlate with the biological output of a cell (Fig.i14) (Bannister and Kouzarides 2011).
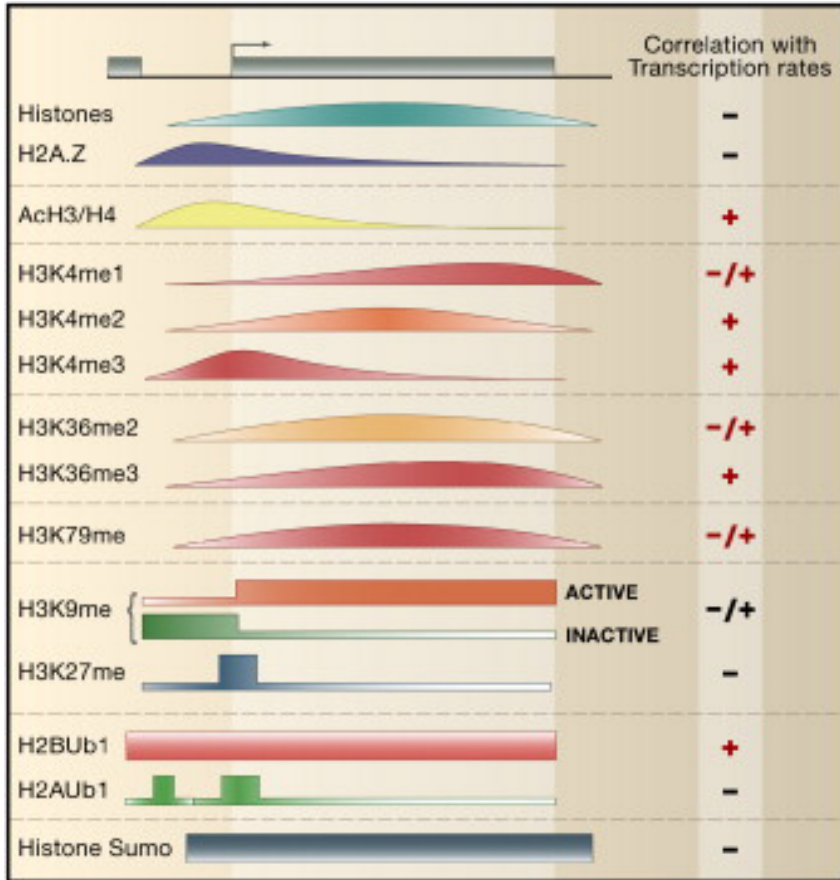
Figure.i13. Regulation of gene transcription by histone PTMs. Representative image of several histone post-translational modifications and their relevance on gene transcription. Remarkably, H3K4me3 is found in the promoter regions promoting the transcription and in contrary H3K27me in the same region promotes repression (B. Li, Carey, and Workman 2007).
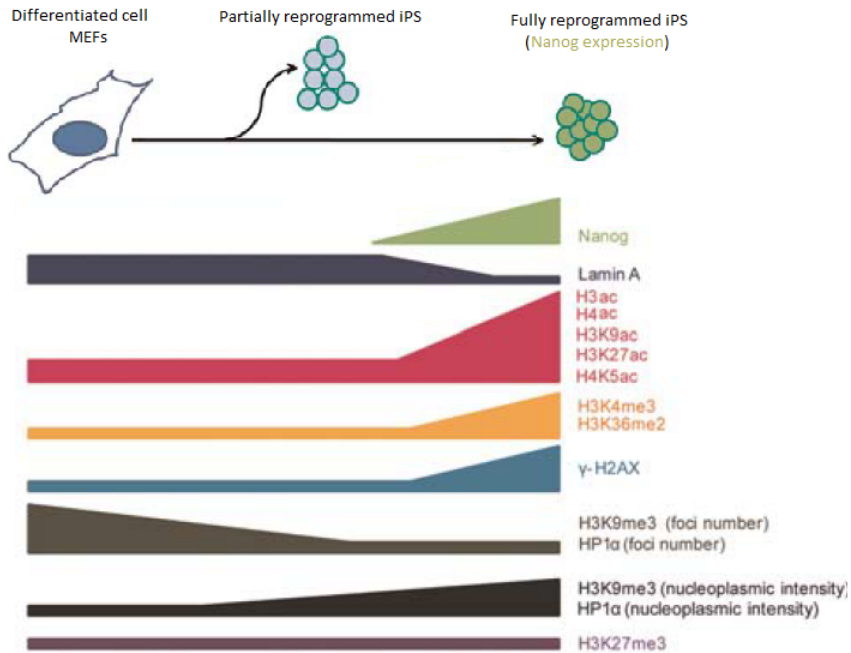
Figure.i14. Post-translational modifications of histones are cell-type specific.
Simplified cartoon of the differences in the histone post-translational modifications during induced-pluripotency stem cell (iPS) reprogramming The levels of the histones post-translational modifications changes in different cell-type and their distribution is implicated in the cellular phenotype of each cell (Cota, Shafa, and E. 2013).

b) **Interplay between marks.**

In addition, a bilateral dialogue exists between most of these modifications. The communication can be either direct, or through chromatin-associated proteins (Fig.i15). Also, this cross-talk can be between modifications in the same histone tail domain (cis) or in histone tail domains of other histones of the same nucleosomes, and from different nucleosomes (trans). An example of the cis communication between epigenetic marks is the phosphorylation of serine 10 in histone 3 (H3S10P) that leads to a previously described

(Mateescu et al. 2004) loss of the H3K9me3. As an example for the trans regulation, there is the phosphorylation of threonine 119 in histone H2A (H2AT119P) promoting lysine acetylation in residues 5 of H4 (H4K5ac) and in residue 14 in H3 (H3K14ac) (Ivanovska et al. 2005).
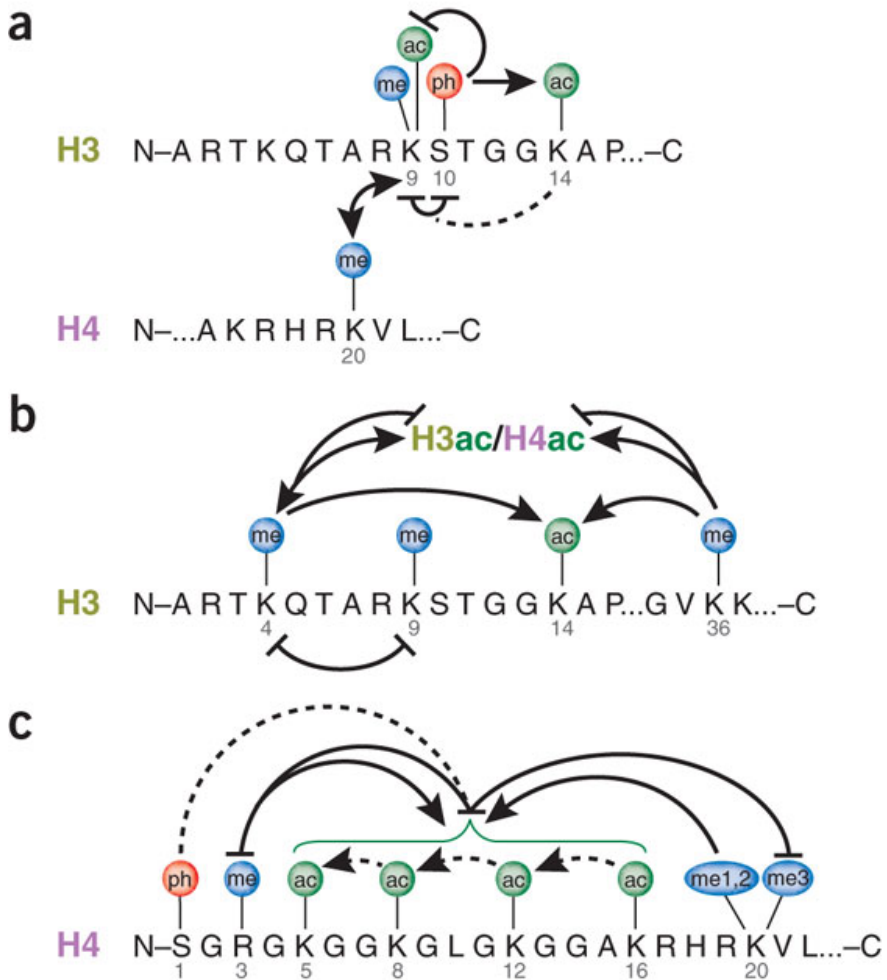
Figure.i15. Interplay between histone post-translational modifications. Exemplary image of three situations of promotions and repressions between the different histone post-translational modifications. (A) Cross-talk (in cis and trans), in the tail domains of histone 3 and 4, between the repressive marks H3K9me and H4K20me and the mitotic one H3S10P. (B) Cross-talk (in cis and

trans) between the active marks (H3K4me, H3K36me, H3K14ac), the repressive mark (H3K9me) and different acetylations in the tail domain of histone 3 and 4. (C) Cross-talk (in cis) in the tail domain of histone 4 between different epigenetics marks. Dotted lines connecting modifications indicate possible cross-talk(Latham and Dent 2007).

This interplay between histone modifications leads to the establishment of pattern of modifications with a specific output (Fig.i16). Hence, those modifications that promote gene transcription are found close to each other and at the same time in a region of the chromatin. For example, when together, H3K4me3, H3K9ac and H4K5ac promote transcription activation (Tollervey and Lunyak 2012), while H3K9me3 and H4K20me3 are found specifically in heterochromatin establishing a repressing environment (Schotta et al. 2004).
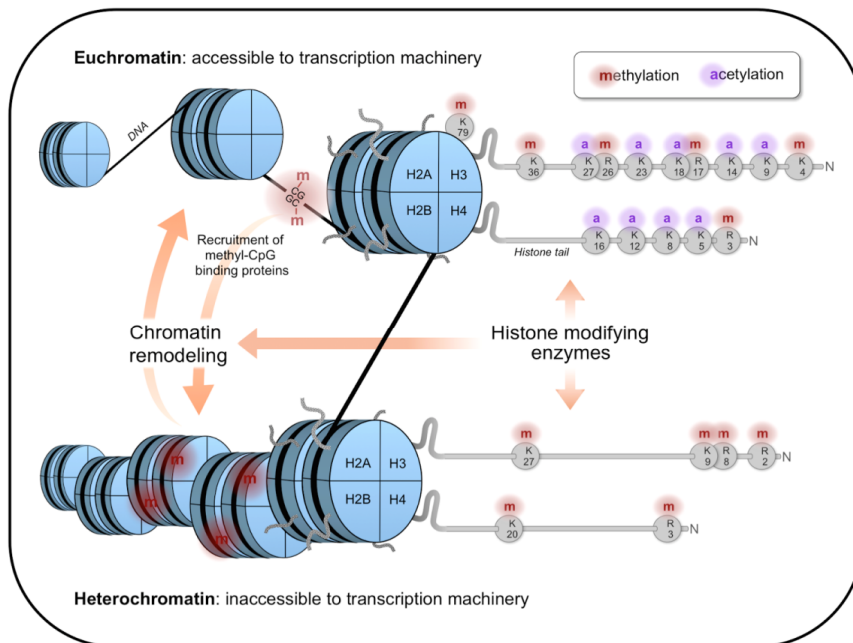
Figure.i16. Differences in histone PTMs between chromatin. Model illustration showing the differences in histone post-translational modifications between euchromatin and heterochromatin (Sam Keating 2016).

However, in a particular case, it is possible to find opposite marks in the same region at the same time (Fig.i17). Bivalent genes are characterized by having both activating and repressing marks in the histone tail domains of their nucleosomes, H3K4me3 (activating mark) and H3K27me3 (repressing mark) being the most described. The presence of these contradictory marks on a promoter is very important in developmental tissues ensuring a tight control of the activation or repression of the developmental genes (Voigt, Tee, and Reinberg 2013; Vastenhouw and Schier 2012). Hence, it gives

robustness to the system ensuring an increased activation threshold and less spurious activation, is suggested to allow low levels of expression of these genes and may have a putative role in stem cell maintenance (Voigt, Tee, and Reinberg 2013).
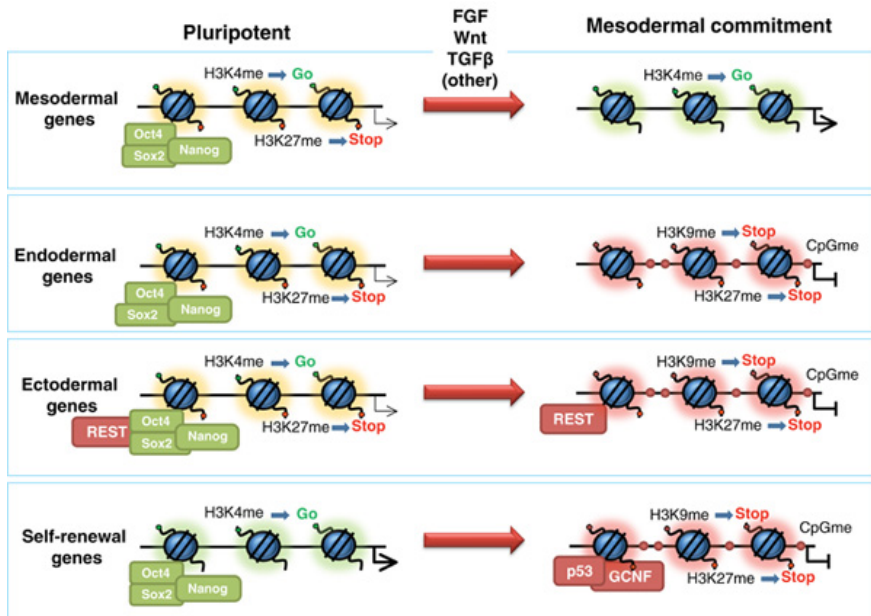
Figure.i17. Paradoxal presence of active and repressive marks in the same genes. Simplified cartoon showing the relevance of having contradictory marks (H3K4me3 an active mark and H3K27me3 a repressive one) in the same regions in pluripotent cells. After the induction of differentiation into a mesodermal commitment, the pluripotent genes are repressed. Then, the mesodermal genes lose the repressive mark and the endodermal and ectodermal genes lose the H3K4me3 (Barrero and Izpisua Belmonte 2008).

c) **Beyond the mark: writers, erasers, readers.**

The tightness of control is not only reflected in the presence or absence of a certain mark, but different players emerge to provide a new level of regulation: the writers, the erasers and the readers (Bannister and Kouzarides 2011) (Fig.i18). Those proteins able to

deposit an epigenetic mark to the histone tail domain are considered as writers. For example, in mammals, lysine 4 methylation in histone 3 (H3K4me3) is deposited by the histone methyltransferase (HMT) family of proteins with non-overlapping consequences by each methyltransferase (H. Wang et al. 2001; Miller et al. 2001). Those proteins that remove an epigenetic mark are considered as erasers. For example, lysine-specific demethylase 1 (LSD1) is able to remove both mono and dimethylated lysine residues 4 or 9 in the histone 3 (Metzger et al. 2005; Shi et al. 2004). Last but not least, those proteins able to recognize and interact with an epigenetic mark in the histone tail domain are considered as readers. HP1 protein, as previously described, is an example of a reader of H3K9me3 (Schotta et al. 2004). Here is important to remark the new step of regulation opened by the cross-talk between the epigenetic mark and the putative reader (Bannister and Kouzarides 2011). Hence, each epigenetic mark will be recognized by a specific domain in the readers shared among all of them (Musselman et al. 2012). For example, H3K9me3 is recognized specifically by the chromodomain in the N-terminal region of HP1α (Jacobs and Khorasanizadeh 2002).
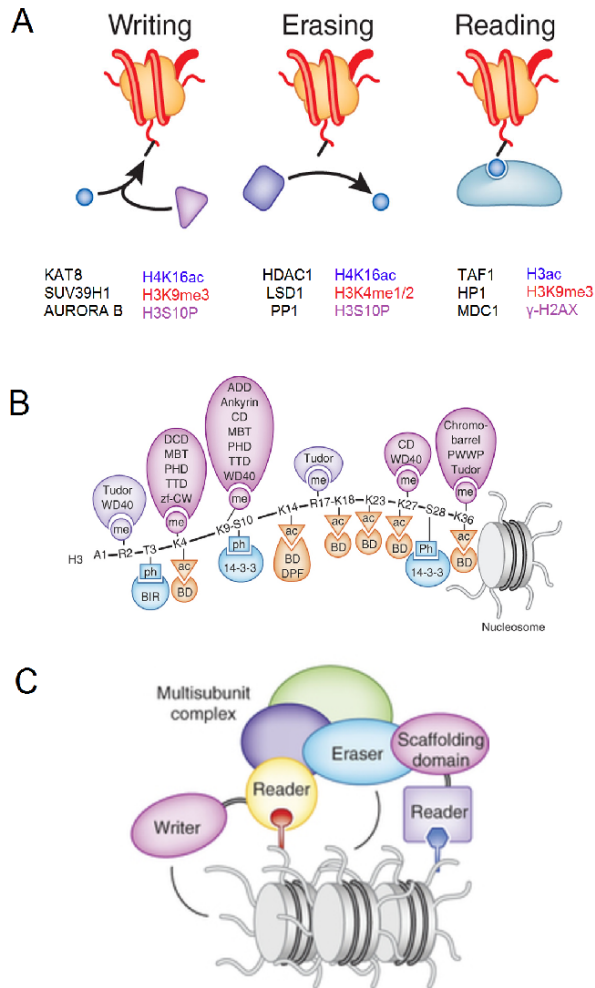
Figure.i18. The players of the histone epigenetic mark. Representative cartoons showing the three different types of proteins involved in the histone modifications. (A) Writers are those proteins able to leave an epigenetic mark on the histone tail. Erasers, those ones able to remove the mark. Readers are those proteins that bind to the mark in order to regulate the chromatin region where the mark is. Some examples of each group are listed and the modification which are related to: acetylation (blue), methylation (red) and phosphorylation (purple). (B) Examples of the different domains able to recognize different epigenetic marks in the tail domain of histone 3. (C) Model cartoon with an increased degree of complexity in order to described the intricacy behind the regulation of histone PTMs (Adapted from (Tarakhovsky 2010; Yun et al. 2011; Musselman et al. 2012)).

Definitely, the three-dimensional architecture of the genome in the nucleus, the chromatin environment, the level of compaction and the different modifications in nucleosomes become different levels of regulation for the correct development of each cell in a complex organism (Fig.i19).
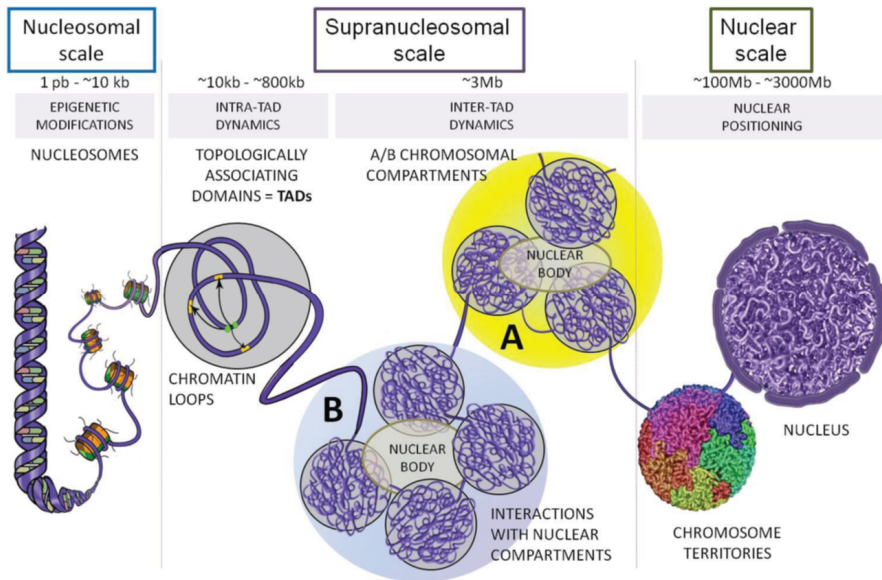
Figure.i19. From the DNA sequence until the genomic organization. Schematic representation that shows the different scales of regulation summarized in the introduction from 1 bp to 3000 megabases (Mb) (Ea et al. 2015)

1.2. A CANCER PERSPECTIVE

1.2.1. Hallmarks of cancer

Cancer is a set of heterogeneous diseases that are driven largely by the accumulation of genetic and epigenetic abnormalities (D

Hanahan and Weinberg 2000) (Fig.i20). Such abnormalities have been considered the major causes of neoplasia and metastasis, but in the last decades, new features have been identified as cancerous events (Douglas Hanahan and Weinberg 2011). Since, these are the most accepted characteristics of a tumoral process:

- Sustaining proliferative signaling

Cancer cells lose the control on the growth-promoting signals leading to an abnormal entrance to the cell growth- and-division cycle. Hence, cancer cells gain a characteristic chronic proliferation. The abnormal activation of oncogenic programs arises as the main responsible pathway, as an example the myelocytomatosis (MYC) protein expression (Dang 2012).

- Evading growth suppressors

Related to the chronic proliferation, there is the inhibition of the tumor suppressor proteins, in order to eliminate a major step of abnormal function in cell behavior. A prototypical example of tumor suppressor are the Retinoblastoma (RB) proteins (Hinds and Weinberg 1994).

- Aerobic glycolysis inhibition

Interestingly, cancer cell switch its metabolic pathway from a normal glucose metabolism that implies mitochondria organelle, to an abnormal glycolysis program, characteristic of anaerobic environments. This described as the "Warburg effect" and as an

"aerobic glycolysis" program may be counterintuitive due to the lower efficiency in ATP production of glycolysis compared to normal glucose metabolism. Hence, the glucose import by cancer cell is dramatically increased. However, the use of glycolytic intermediates in other biological pathways could be an explanation. A Warburg-like effect takes place in some rapidly dividing embryonic tissues (Gatenby and Gillies 2004).

- Enabling replicative immortality

In a highly replicative population as cancer cell, two main barriers have to be overcome: senescence induction and replication crisis. Rarely normal cells are able to replicate more than a limited number of cell cycles. Cancer cells deregulate telomerase protein in order to break these two-crucial anticancer defenses. Hence, telomerase hyperactivity ensures the enlargement of telomeric regions in order to avoid its lose during high replication ratios (Shay and Wright 2011).

- Inducing angiogenesis

Strikingly, a major step that cancer cells need to bypass is the lack of vascularization inside the tumor. Thereby, cancer cell promotes an angiogenic program to ensure the presence of oxygen and nutrients for the tumor. Consequently, factors as vascular endothelial growth factor-A (VEGF-A), an angiogenic inducer, are promoted (Nishida et al. 2006) and factors as thrombospondin-1 (TSP-1) an angiogenic inhibitor, repressed (Lawler and Lawler 2012).

- Genome instability and mutation

Another characteristic of tumor cells is a characteristic pattern of mutations on DNA that underlie cancer progression. This has been highlight in the last decades thanks to the high-throughput sequencing techniques (Negrini, Gorgoulis, and Halazonetis 2010).

- Resisting cell death

As cancer cells accumulate mutations, they, definitely, need to hijack the directed cell death program also called apoptosis. Hence, the suppression of apoptosis inducers as for example the Apoptosis regulator BAX (Degenhardt et al. 2002) and/or the induction of antiapoptotic proteins as Bcl-2 family of proteins (Delbridge and Strasser 2015) are characteristic features of cancer cells.

- Avoiding immune destruction

Tumor in tissue may be considered as a strange organ for the immune system of human organism. Accordingly, both the innate and adaptive cellular arms of the immune system may be able to contribute significantly to the tumor eradication (Marcus et al. 2014). Numerous of immune suppressive cytokines were reported to be released in the tumor environment: TNFα, for example (Landskron et al. 2014).

- Tumor-promoting inflammation

Despite the effort of cancer cells in evading the immune destruction, clear evidences have arisen in the last years of an

inflammation promotion in tumorigenic environment. Thereby, inflammatory reactive species may display tumorigenic features as growth factors and proangiogenic products (Whiteside 2008).

- Activating invasion and metastasis

An important characteristic of cancer cells, particular in carcinomas arising from epithelial cells, is the capacity to invade the surrounding tissue and to migrate to different organs of the organism and the formation of distant metastasis from the primary tumor. This program is known as epithelial-to-mesenchymal transition (EMT) (Yang and Weinberg 2008).
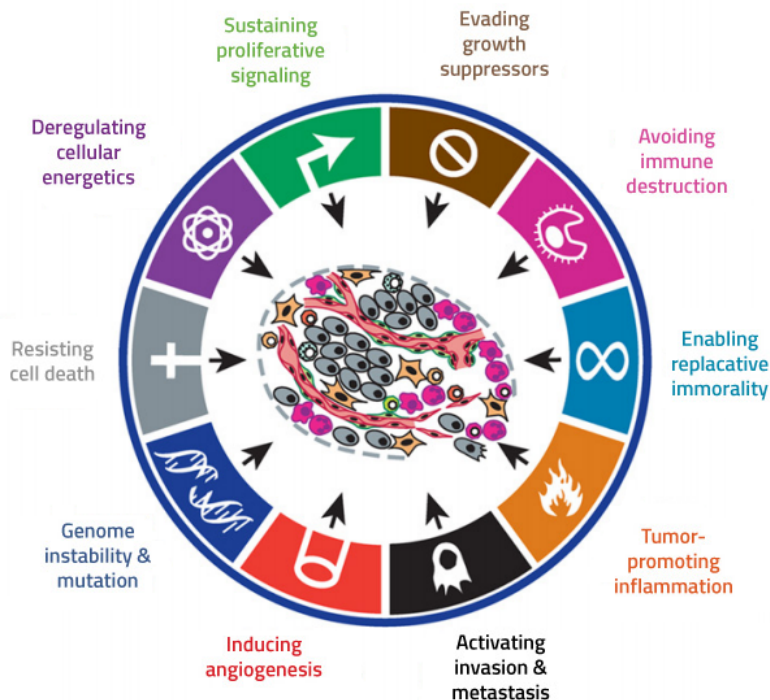
Figure.i20. The hallmarks of cancer. Representative cartoon of the summarized characteristics of a cancer disease(Douglas Hanahan and Weinberg 2011)

1.2.2. Epithelial-to-mesenchymal transition (EMT)

a) **A transition of properties**

Epithelial-to-mesenchymal transition (EMT) is a key process for the embryonic development of an organism (Thiery et al. 2009; Yang and Weinberg 2008; X. Ye and Weinberg 2015; Nieto et al. 2016; Diepenbruck and Christofori 2016; Cebrià-Costa et al. 2014). EMT consists in the phenotypic change of an epithelial cell to a mesenchymal cell (Fig.i21). Epithelial cells are characterized by being organized in an epithelia establishing close contacts with neighbor cells. These contacts, in addition, establish an apicobasal axis of polarity through the sequential arrangement of adherent junctions, desmosomes and tight junctions. Epithelial cells organize as a unit by maintaining a global communication through gap junctional complexes. Epithelia remain separated from surrounding stroma by a basal lamina. On the other hand, mesenchymal or stromal cells are found in the stroma surrounded by the extracellular matrix (ECM). They are characterized by being loosely organized and by the absence of any kind of polarity. Mesenchymal cells are able to migrate along the ECM by extending filopodia through the loose matrix to move in response to chemical or mechano-gradients. Hence, the conversion of epithelial cells to mesenchymal involves the loss of the epithelial characteristics and the acquisition of migratory properties. EMT comprises the gradual remodeling of epithelial cell architecture losing their epithelial cell–cell junctions and apicobasal cell polarity and convert to the low proliferation state with spindle-like cell shape, front-back polarity and with increased cell migration, invasion and survival of a mesenchymal

cell. Interestingly the inverse transition has been largely described knew as mesenchymal-to-epithelial transition (MET) (Gunasinghe et al. 2012).
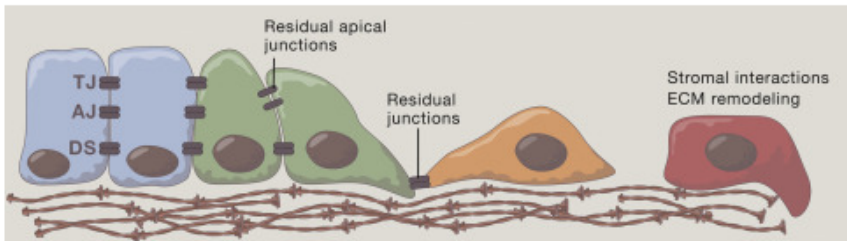
Figure.i21.Epithelial-to-mesenchymal transition (EMT) I. Exemplary cartoon of the continuous differentiation of the cells that are undergoing an EMT event. From left to right, epithelial cells lose the apicobasal polarity and the cell-cell contacts. Then, after pass throughout different intermediate states, the cells achieve a mesenchymal phenotype with a front-back polarity and increased cell-matrix contacts. TJ (Tight junction); AD (Adherent junction); DS (Desmosome). (Nieto et al. 2016).

The EMT process takes place in a number of cellular contexts during the embryonic development as well as in adult tissues (Fig.i22). For example, during gastrulation, epithelial cells from the epiblast undergo an EMT in order to delaminate and form the embryonic tissues of the gastrula. Depletion of different players involved in the EMT blocks the gastrulation (Lomelí, Starling, and Gridley 2009). Indeed, the similarities between carcinoma progression and embryonic development establish EMT as a driver of tumors. Hence the EMT will be responsible of distant metastasis formation from a primary tumor. However, EMT also participates in other events important for tumor progression, for example resistance to cell death (Vega et al. 2004), resistance to

chemotherapy (Fischer et al. 2015) and stem cell-like phenotype (Pandian et al. 2015).
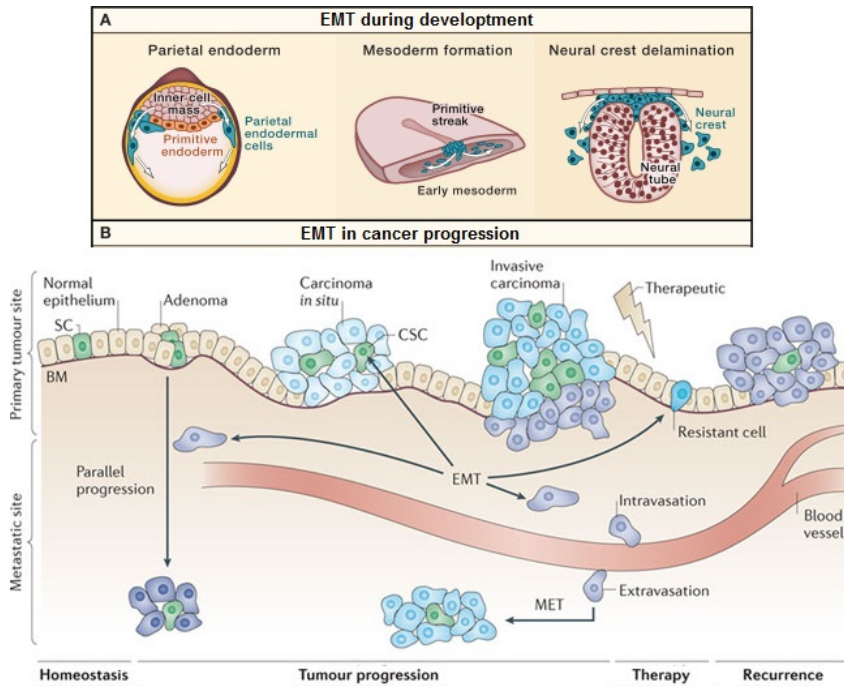
Figure.i22. Epithelial-to-mesenchymal transition (EMT) II. Schematic representation of different EMT processes. (A) Three examples of EMT during normal embryonic development. Firstly, even before the implantation of the blastocyst, the formation of the parietal endoderm requires EMT (left). Then, the mesendodermals progenitors undergo a second EMT in the gastrulation process (center). Lately, the delamination of the neural crest cells from the dorsal neural tube also requires an EMT process (right). (B) EMT events during cancer progression. (Adapted from (Thiery et al. 2009; Craene and Berx 2013)).

b) **The SNAIL protein family**

Accordingly, many different players are involved in EMT cascade signal. Definitely, the SNAIL protein family of transcription factors are the most described. This family contains three different proteins: SNAIL protein (SNAI1), SLUG (SNAI2) and SMUC (SNAI3) (Nieto 2002; de Herreros et al. 2010). They are characterized by

three different domains (Fig.i23): the zinc-finger C-terminal domain, which contains from four to six C_2H_2 type zinc fingers and is the domain able to bind to the E-box 5'-CACCTG-3', the central part that contains post-translational modifications and is involved in the protein stability and localization and the N-terminal region with a SNAG subdomain where interaction with other proteins refine the role of SNAI1 in the cell.
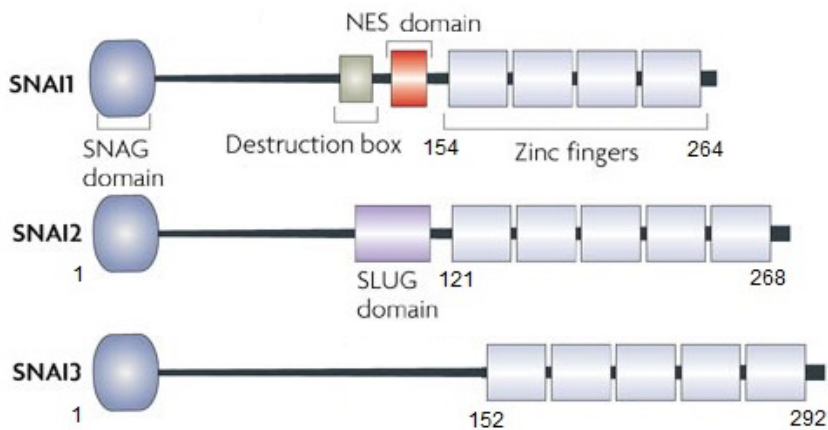
Figure.i23. SNAIL protein family. Representative cartoon of the different proteins that form the SNAIL family of proteins. (Adapted from (Héctor Peinado, Olmeda, and Cano 2007)).

The main function of this family of proteins is the repression of the epithelial phenotype and promotion of the mesenchymal one (Fig.i24). They are able to bind to the promoter of E-cadherin gene (cdh1) and repress its expression (Batlle et al. 2000). The repression of CDH1, a typical adhesion molecule, impairs the epithelia structure by the loss of cell-cell contacts. SNAI1 expression is tightly controlled by many different pathways as TGF-β family of

40

proteins (Hector Peinado, Quintanilla, and Cano 2003; Choi, Sun, and Joo 2007), WNT signaling (Yook et al. 2005), NF-κB pathway (Julien et al. 2007), hypoxia (Liu et al. 2011; L. Zhang et al. 2013), fibroblast growth factors (FGFs), microRNAs (miRNAs) (Díaz-López, Moreno-Bueno, and Cano 2014) and strikingly, by itself in a negative loop, limiting its own expression (Peiró et al. 2006). Furthermore, SNAI1 stability is regulated by different proteins in order to control its levels in the cell. Since, for example, phosphorylation of SNAI1 by the glycogen synthase kinase 3β (GSK-3β) exports SNAI1 to the cytoplasm favoring its degradation (B. P. Zhou et al. 2004; Schlessinger and Hall 2004). Interestingly, LOXL2 is able to interact with the SNAI1 in the N-terminal and stabilize it. In addition, this interaction allows the directed deamination activity of LOXL2 in the SNAI1 target promoters (Herranz et al. 2016).
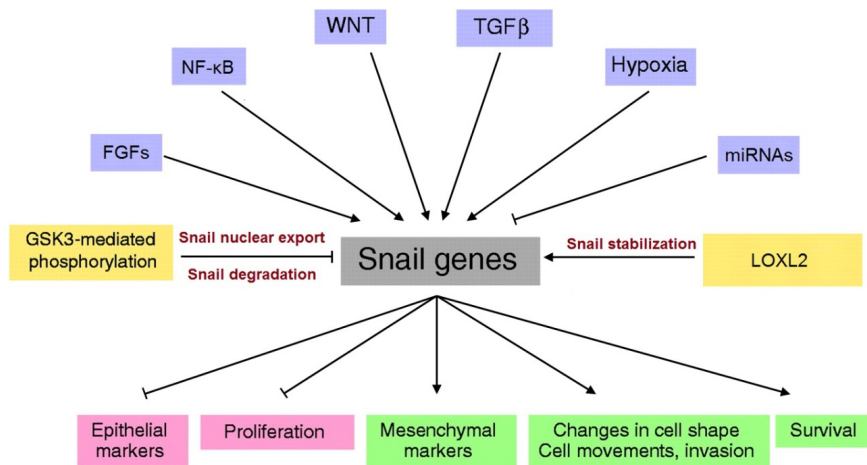
Figure.i24. Regulation network of SNAIL protein family. Simplified representation of the described regulators of the SNAIL genes. (Adapted from (Barrallo-Gimeno and Nieto 2005)).

1.2.3. DNA-DAMAGE

As previously seen, cancer cell is characterized by the accumulation of mutations in its DNA sequence. DNA lesions and mutations can be induced by endogenous sources, such as defects during DNA replication or cell division, metabolic by-products, or exposure to damaging agents, UV light, or irradiation (Jackson and Bartek 2009) (Fig.i25). In fact, a normal cell may suffer a total of about 10^5 lesions per day (Lindahl 1993). Each different inductor of DNA damage cause different lesions to DNA (Houtgraaf, Versmissen, and van der Giessen 2006; Ciccia and Elledge 2010).
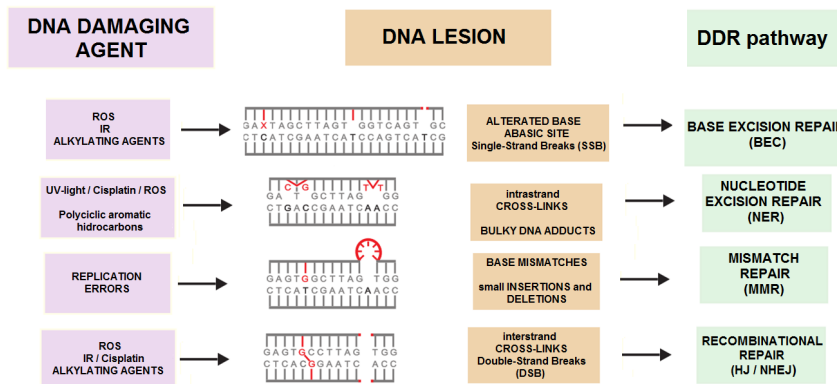
Figure.i25. Regulation network of SNAIL protein family. Summary of the most common types of DNA lesions. There are list the different damaging agents for each DNA lesion (top) and the DNA damage response (DDR) mechanism used to resolve it (bottom). ROS (Reactive oxygen species); IR (ionizing radiation) (Houtgraaf, Versmissen, and van der Giessen 2006).

In order to fix these lesions, many processes evolved for each type of lesion (Jeggo, Pearl, and Carr 2015). These processes are known as DNA damage response (DDR) pathways. Moreover, the repair of

the different lesions must occur in a chromatin context (Fig.i26). There is increasing evidence that the pathways that repair these lesions are also regulated by histone modifications and chromatin remodelling (Gursoy-Yuzugullu, House, and Price 2016). In fact, cancer genome sequencing studies have shown substantial variation in somatic mutation rates, with an increase in the rates in heterochromatin (closed chromatin) as compared with euchromatin (open chromatin) (Hodgkinson, Chen, and Eyre-Walker 2012; Schuster-Böckler and Lehner 2012). Furthermore, it has been recently reported that DNA mismatch repair (MMR) is more efficient in euchromatin genomic regions than in heterochromatin, such that no mutations accumulate in these open genomic regions (Supek and Lehner 2015). Accordingly, these differences could be determined by different accessibility to DNA repair complexes (Goodarzi et al. 2008; Sabarinathan et al. 2016) or by variations in the ability to activate the DDR (Misteli 2007).

A
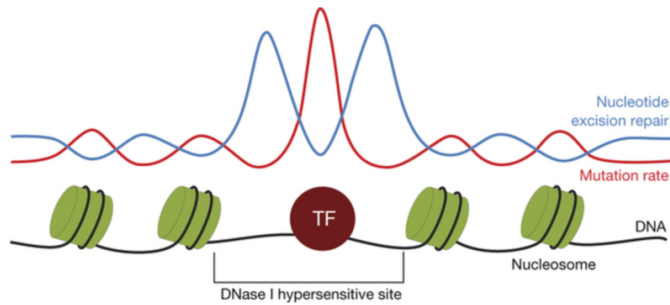
B
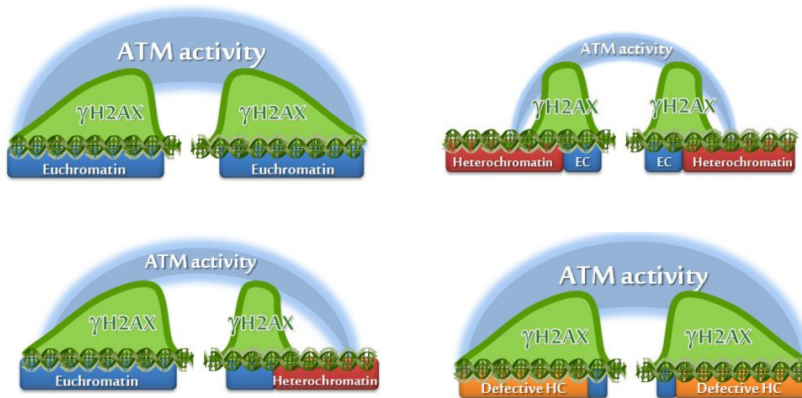
Figure.i26. The chromatin accessibility alters the DDR. Schematic drawings explaining how the chromatin accessibility present differences in the DDR signaling. (A) Recently, the presence of a transcription factor has been described as in impediment to the recruitment of NER machinery causing an increase in the mutation rates in the transcription factor binding site. (B) The activation of the ATM response is smaller in the heterochromatin regions due to the highly compaction of the chromatin. In fact, altered heterochromatin enhances the ATM response even more than the euchromatin levels (Goodarzi and Jeggo 2012; Sabarinathan et al. 2016).

One of the more dramatic lesion on the DNA are the double-strand breaks (DSBs) (Houtgraaf, Versmissen, and van der Giessen 2006; Watts 2016) (Fig.i27). These lesions pose serious threats to genetic

integrity and cell viability, since, if not identified and repaired, they can lead to insertions or deletions, or gross chromosomal rearrangements. Hence, they cause a signaling response that can activate cell cycle checkpoint arrest and/or apoptosis or senescence. Briefly, DSB needs to be detected, then the signal is amplified and finally there are the effectors mechanism to solve the DSBs (Khanna and Jackson 2001; A. Shibata and Jeggo 2014). The major DSB sensor mechanism is the MRE11/RAD50/NSB1 (MRN) complex. When this complex finds a lesion, it recruits via the C-terminus of NBS1 the two main proteins in charge of the signal amplification: the phosphatidylinositol 3- kinase-related kinases, ataxia telangiectasia mutated (ATM) protein and ataxia telangiectasia and Rad3-related (ATR). Despite both proteins get activated after a DNA lesion, ATM activation is more robust in the DSB signaling than ATR which plays a main role in single strand break (SSB) repair. The ATM/ATR recruitment promotes their autophosphorylation and activation. Swiftly, ATM phosphorylates the serine 139 of H2A.X (H2AXS139P or γ-H2A.X), a histone variant of H2A. This phosphorylation is a key step for the recruitment to chromatin of mediator of damage-checkpoint 1 (MDC1). Hence, MDC1 recruits two ubiquitin-ligase proteins RFN8 and RFN168 which activity is necessary for the correct recruitment of p53 binding protein 1 (53BP1). The recruitment of all these players stabilizes each one of them and favours their activity. In fact, the absence of some of these proteins do not alter the recruitment of the others although impairs their stability and the DDR (Atsushi Shibata et al. 2010; Fernandez-capetillo et al. 2002).
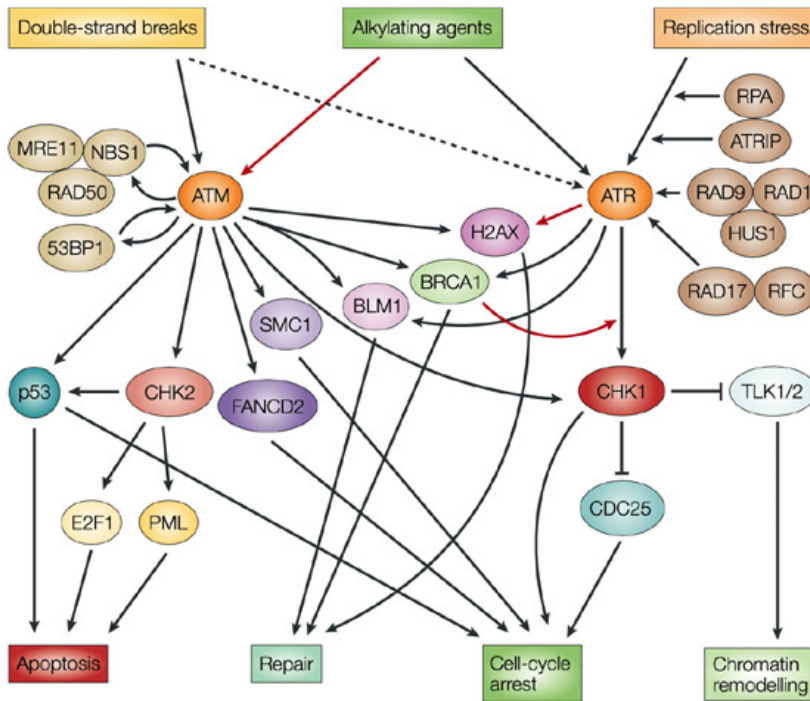
Figure i27. DSB repair: Detection, amplification, response. Representative cartoon of the complex network after a DNA lesion. Focusing on the DSB response, the MRN complex detects the lesion, recruits ATM and ATR proteins to amplify the signal and regulate the different responses of the cell (B.-B. S. Zhou and Bartek 2004).

Besides, ATM/ATR control the cell cycle checkpoints in order to arrest the cell cycle progression for an efficient repair (Fig.i28). These arrests take place between the different phases of cell cycle and three different ones have been reported: G1/S checkpoint, intra-S checkpoint and G2/M checkpoint (Deckbar et al. 2010; Lukas, Lukas, and Bartek 2004). The G1/S checkpoint is activated by the direct or indirect phosphorylation of p53 and its regulatory protein MDM2 by ATM. This promotes p53 stabilization and transcription

of its target genes, among them the cyclin-dependent kinase (CDK) inhibitor p21, which blocks the Retinoblastoma (Rb) phosphorylation indispensable for the progression along the G1 phase. Remarkably, a permanent arrest of heavily damaged cells influences the genomic stability of those cells (Brunton et al. 2011; A. Shibata and Jeggo 2014). The intra-S checkpoint activates when the replicative forks common of the transcription process collapse. The intra-S checkpoint relies on the ability of the replication protein A (RPA) to protect the single strand DNA (ssDNA) of a collapsed fork and the activation of ATR-cascade signaling (Smith-Roe et al. 2013; Iyer and Rhind 2017). The G2/M checkpoint controls the entrance of the cell into mitosis (A. Shibata and Jeggo 2014; Fernandez-capetillo et al. 2002). This checkpoint is regulated by the checkpoints kinases CHK1 and CHK2. These kinases are activated by the phosphorylation of ATR and ATM respectively. The checkpoints kinases regulate the levels of CDC25 phosphatase delaying the necessary dephosphorylation of the cyclin dependent kinase 1 (CDK1).
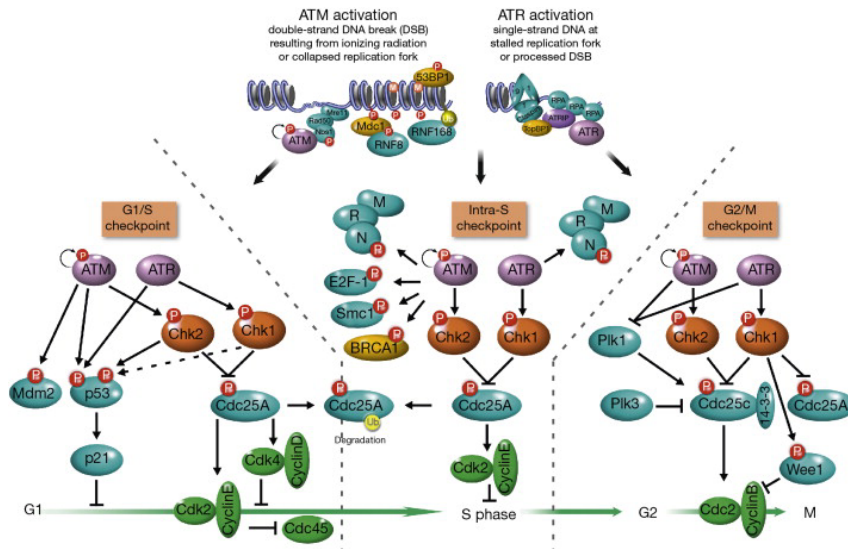
Figure.i28. **DSB repair: Stop at the checkpoint.** Schematic picture of the different checkpoints that a damaged cell activates(Weitzman and Wang 2013).

Finally, the different arrests allow different and specific strategies for the repair of the DSB. Two main pathways turn on to repair DSB: the non-homologous end-joining pathway (NHEJ) and the homologous recombination (HR) (Fig.i29). NHEJ is faster than HR but more prone to errors because it uses short homologous DNA sequences for repair instead the large templates used by HR (Shaltiel et al. 2015). Under the G1/S checkpoint, the main mechanism used to repair DSB is the non-homologous end-joining pathway. The NHEJ pathway relies on the INO80 complex recruitment by γ-H2A.X (Cairns 2004). The INO80 complex is a chromatin remodeler ATP-dependent complex and its function in the DNA repair allows a more accessible environment promoting a more "open" state by different acetylations in lysine 14 of histone 3 (H3K14ac) and also serves as a dock for the repair proteins like

DNA ligase (Van Attikum et al. 2004; Morrison et al. 2004). Lastly, DNA polymerases Pol λ and Pol μ fill in the gaps of the DSB and DNA ligase IV and its cofactor XRCC4 performs the ligation step of the DNA repair (Davis and Lin 2011). During the G2/M cell checkpoint activation, all the genetic material is replicated in order to undergo the mitosis. Hence, cells use the sister chromatid or the homologous chromosome as a template in order to repair the lesions in a process known as homologous recombination (HR) (Ceccaldi, Rondinelli, and D'Andrea 2016). HR requires homology search and strand invasion mediated by the recombinase RAD51 protein and the dsDNA motor protein Rad54. Hence, it generates a D-loop structure that allows polymerases to refill the gap of the DSB repairing the lesion. HR is typically error-free even though completion of HR often requires error-prone polymerases (Heyer, Ehmsen, and Liu 2010).
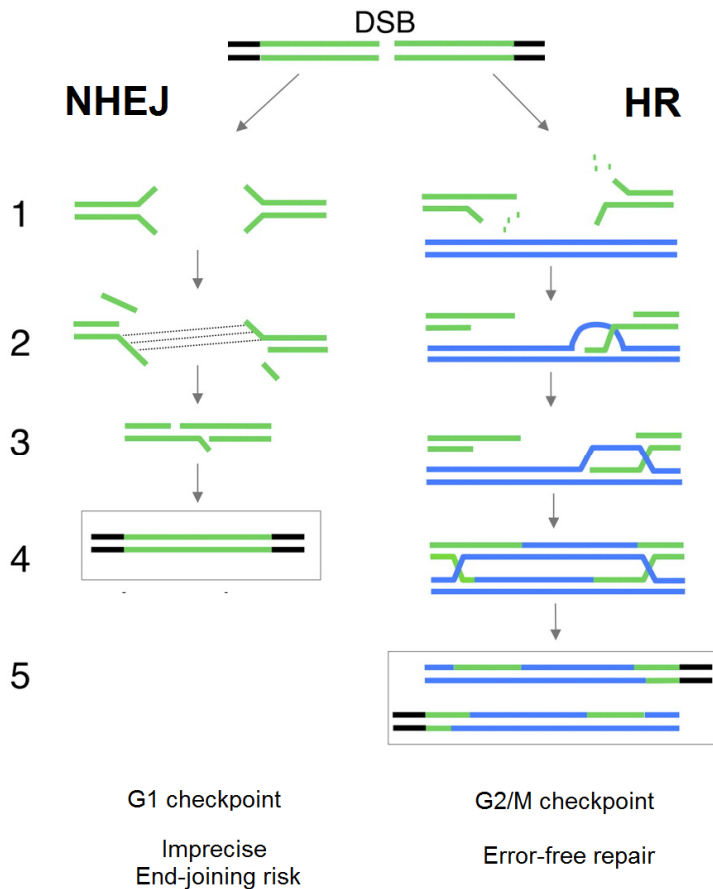
F**igure.i29. DSB repair: Repairing the lesion.** Simplified diagram of the two main pathways in order to repair the DSB (adapted from (Miura et al. 2012)).

It should be noted that all the processes previously described cannot be explained without taking into consideration the chromatin structure. Indeed, increasing evidence suggests that the higher-order chromatin structure affects DSB repair and signaling (Gursoy-Yuzugullu, House, and Price 2016). For example, chromatin is decondensed after DSBs, and this phenomenon is actively regulated by the DDR pathway (Ziv et al. 2006). Moreover, the DDR is

amplified when chromatin is in an "open" state, as shown in histone H1–depleted embryonic stem cells (Murga et al. 2007). As a consequence, relaxation of chromatin might facilitate genomic surveillance by enabling faster access of DDR factors to the DSBs (Gursoy-Yuzugullu, House, and Price 2016; Watts 2016).

1.2.4. Breast Cancer as a model

One type of cancer where the processes of EMT and DDR have been studied the most is breast cancer. Breast cancer is the leading cause of cancer death in women worldwide (Koren and Bentires-Alj 2015). Moreover, it is also the most diagnosed one (Kumar and Aggarwal 2016). Although a lot of information has been reported about this type of cancers (Koren and Bentires-Alj 2015; Kumar and Aggarwal 2016; Lechner, Boshoff, and Beck 2010; Palma et al. 2015), they can be divided into at least six different major groups with different subgroups, based on different histopathological parameters and molecular profiling such as receptor presence (estrogen-receptor, progesterone-receptor, and/ or ERBB2/HER2): normal like, luminal A, luminal B, HER2 enriched, claudin low, and basal-like (BLBC) (Chiorean, Braicu, and Berindan-Neagoe 2013; Santagata and Thakkar 2014) (Table.1 and Fig.i30).

Classification	Immunoprofile	Other characteristics	Example cell lines
Normal breast like	Adipose tissue gene signature[+]		
Luminal A ~40%	ER[+] / PR^{+/-} / HER2^{-}	Ki67 low, endocrine responsive, often chemotherapy responsive	MCF-7, T47D, SUM185
Luminal B ~20%	ER[+] / PR^{+/-} / HER2^{+}	Ki67 high, usually endocrine responsive, variable to chemotherapy. Her2^{+} trastuzumab responsive	BT-474, ZR-75
Her2 10-15%	ER[-] / PR[-] / HER2^{+}	Ki67 high, trastuzumab responsive, chemotherapy responsive	SKBR3, MDA-MB-453
Basal 15-20%	ER[-] / PR[-] / HER2^{-}	EGFR[+] and/or cytokeratin 5/6[+], Ki67 high, endocrine nonresponsive, often chemotherapy responsive	MDA-MB-468,SUM190
Claudin-low 10-15%	ER[-] / PR[-] / HER2^{-}	Ki67, E-cadherin, claudin-3, claudinins -4 -7 low. Intermediate response to chemotherapy.	MDA-MB-231, BT549, Hs578T, SUM1315

Table.1. Molecular classification of human breast carcinoma. Summary of the different types of breast cancer. The percentages under each group indicate their grade of incidence. The two shaded groups compose the TNBC subtype. Ki67 is a marker of proliferation. Trastuzumab is a humanized monoclonal antibody that targets the Her2 receptor and blocks the proliferation of the Her2^{+} cells. ER (Estrogen receptor); PR (Progesterone receptor). (Adapted from (D. L. Holliday and Speirs 2011)).

Considering, there is compelling evidence for the existence of carcinoma cells with a mesenchymal phenotype in human breast cancer as well as in mouse breast cancer models (Bill and Christofori 2015). Furthermore, the functional manipulation of key EMT players in breast cancer has provided clear evidence for a causal involvement of EMT-inducing or blocking factors in metastasis. For example, as previously explained, the importance of CDH1 protein levels in breast cancer under SNAI1 regulation highlights the role of EMT and MET programs (Gunasinghe et al. 2012; de Herreros et al. 2010). Likewise, for those breast cancers that are intrinsically chemotherapy resistant, chemotherapy induces a mesenchymal phenotype rather than alter the genetic tumor cell diversity (Almendro et al. 2014). Remarkably, breast cancer has been used largely to study the effects and relevance of DNA damage and chemotherapy in the different breast cancer cells (Davis and Lin 2011; J. and Li 2012). For example, mutations in the Breast cancer type 1 susceptibility protein (BRAC-1) (Hall et al. 1992), a crucial protein in the cell cycle arrest after ionizing radiation, is responsible for approximately 40% of sporadic breast cancers and more than 80% of inherited breast and ovarian cancers (J. and Li 2012). Understanding the mechanisms underlying the chemotherapy resistance utilized by cancer cell populations may become basic in the development of efficient treatments in breast cancer patients chiefly with poor prognosis such as TNBC or BLBC (Davis and Lin 2011).
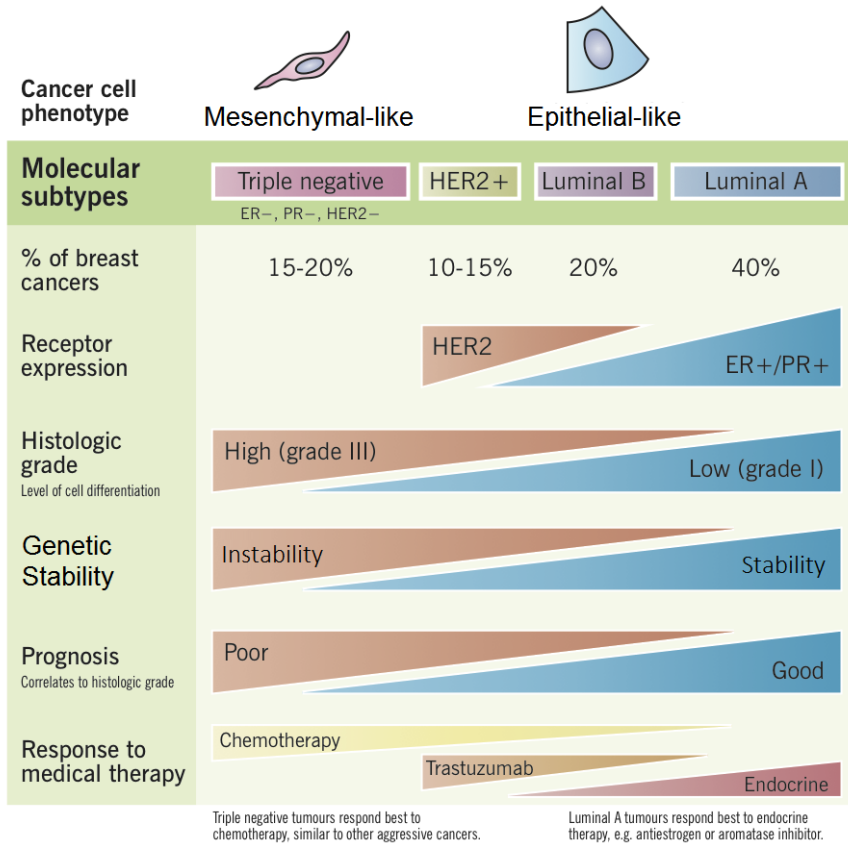
Figure.i30. Molecular classification of human breast carcinoma. Schematic cartoon highlighting the grade of differentiation, the genetic stability, the prognosis and the response to the medical therapy. (Adapted from (Sims et al. 2007; Rossi, Chaudry, and Wong 2012)).

1.3. A LOXL2 PERSPECTIVE

1.3.1.   LOX FAMILY OF PROTEINS

a)   **Structure**

Lysyl oxidase like-2 protein (LOXL2) belongs to the family of the lysyl-oxidaze (LOX) proteins. The LOX family contains 5 different paralogues: lox (LOX), loxl1 (lysyl oxidase like-1, LOXL1), loxl2 (lysyl oxidase like-2, LOXL2), loxl3 (lysyl oxidase like-3, LOXL3), and loxl4 (lysyl oxidase like-4, LOXL4) (Iturbide, García De Herreros, and Peiró 2015). The expression of the members of LOX family proteins is tightly controlled during normal development and in the adult tissues suggesting different roles for each member of the family (Iturbide, García De Herreros, and Peiró 2015; Hein et al. 2001; Akiri et al. 2003). However, different diseases present mutations and abnormalities in the expression of the LOX family of proteins family, for example fibrotic disorders (Byers et al. 1980; Khakoo et al. 1997), cardiovascular diseases (Bonnans, Chou, and Werb 2014) and cancer (Herranz et al. 2016; Ahn et al. 2013; Hase et al. 2014; Seong Park et al. 2016).

The main characteristic of the LOX family members is a conserved carboxy-terminal (C-terminal) amine oxidase catalytic domain, which contains a His-X-His-X-His copper binding motif and a lysine tyrosylquinone (LTQ) cofactor (Fig.i31). Although sharing more than a 50% of homology in the C-terminal catalytic domain the family can be separated into two different groups based on their amino-terminal (N-terminal) structure (Moon et al. 2014; L. Wu

and Zhu 2015). On one hand we find LOX and LOXL1 proteins that contain an small highly basic propeptide at their N-terminal region and on the other hand, LOXL2, LOXL3 and LOXL4 each contain four scavenger receptor cysteine-rich (SRCR) domains in the N-terminal, that are suggested to act as platforms for the interaction with other proteins and could modulate the catalytic activity (Barker, Cox, and Erler 2012; Lugassy et al. 2012).
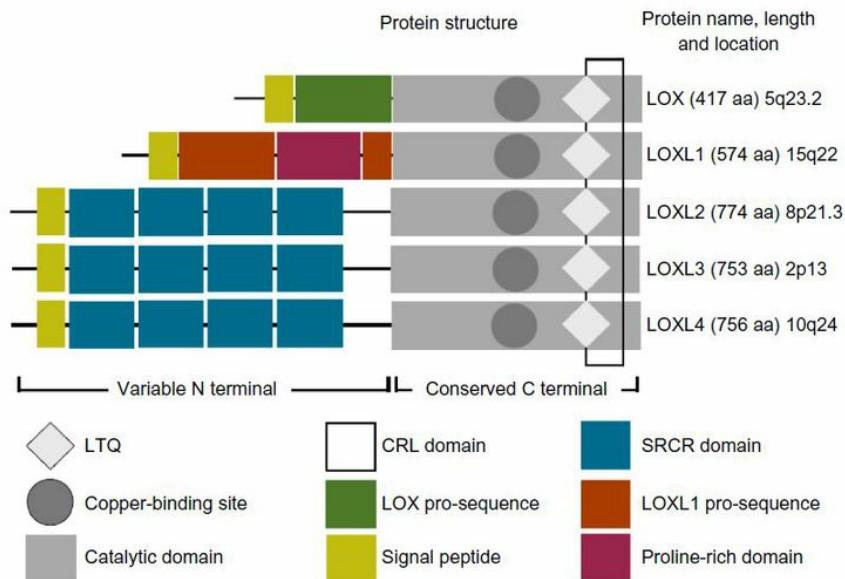
Figure.i31. LOX family of proteins: Structure. Model picture of the structure of the different members of the LOX family. All of them share the same C-terminal domain with the copper binding domain, the lysine tyrosylquinone (LTQ) cofactor residues, and the cytokine receptor‑like (CRL) domain. Instead, their N‑terminal are variable, LOX and LOXL1 contain pro‑sequences, especially the proline‑rich region in LOXL1. LOXL2‑4 contain four scavenger receptor cysteine‑rich (SRCR) domains that are thought to be involved in protein‑protein interactions. (Mayorca-Guiliani and Erler 2013).

b) **Activity**

The primary function for LOX family of proteins is the covalent crosslinking of collagens and elastin in the extracellular matrix (ECM), essential for ECM formation and stiffness. Indeed, LOX and LOX-like proteins catalyze an oxidative deamination of the epsilon-amino group of lysines and hydroxylsines thereby generating an aldehyde group (Siegel 1974; Yamauchi and Sricholpech 2012) (Fig.i32). Being highly reactive, these aldehyde groups can spontaneously condense with other aldehyde groups or with epsilon-amino groups of lysine residues to generate the intra- or intermolecular crosslinkages (Byers et al. 1980; Williamson and Kagan 1986; Khakoo et al. 1997; Hase et al. 2014; Yamauchi and Sricholpech 2012). Despite this extracellular role, more evidences suggested important intracellular roles for the LOX family of proteins (Herranz et al. 2016; Iturbide et al. 2014; Smith-Mungo and Kagan 1998; L. Ma et al. 2017).
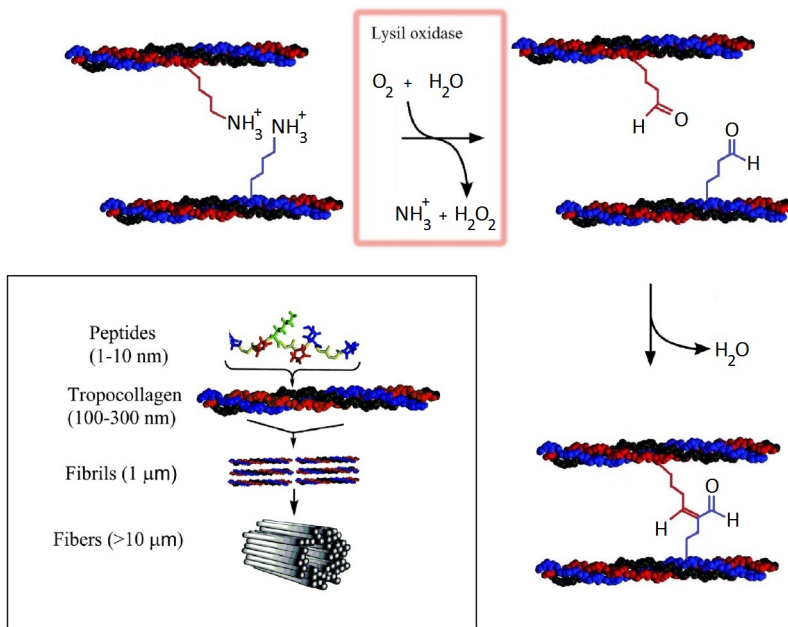
Figure.i32. LOX family of proteins: Extracellular activity. Simplified cartoon showing the activity of LOX family of proteins in the extracellular matrix during the crosslink of collagen fibers. Squared, in the bottom left, all the steps in the collagen formation (Adapted from (Domene et al. 2016)).

LOXL2 protein has been reported to interact with SNAIL 1 (SNAI1) protein, the main inductor of epithelial-to-mesenchymal transition (EMT). This interaction prevents the degradation of SNAI1 by GSK3β, leading to reduced expression of CDH1 (Héctor Peinado, Portillo, and Cano 2005). In addition, LOXL2 also regulates the expression and activity of two well-characterized inductors of cellular invasion: tissue inhibitor of metalloproteinase-1 (TIMP1) and matrix metalloproteinase-9 (MMP9) (Barker et al. 2011). Moreover, previous work in our group described an important role for LOXL2 oxidizing TAF10 protein in embryonic

stem cells after neuronal induction by retinoic acid. The deamination of TAF10 disassembles the TFIID complex repressing the expression of the pluripotency genes (Iturbide et al. 2014) leading to a correct neuronal development (Fig.i33)

Recently, a new activity for the LOXL3 has been described inside the nucleus. LOXL3 deacetylates STAT3 protein inhibiting its dimerization and the transcription of the STAT3 target genes. This LOXL3-STAT3 regulation affects the cellular differentiation of T-cell differentiation in inflammatory response (L. Ma et al. 2017).
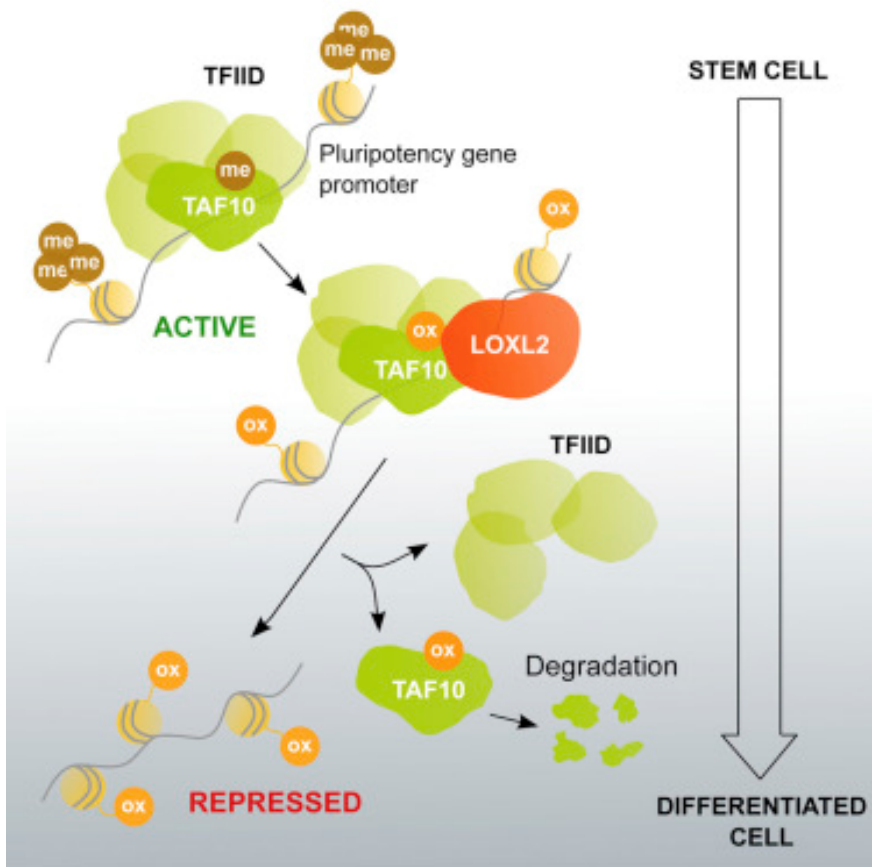
Figure.i33. LOXL2 Oxidizes Methylated TAF10 and Controls TFIID-Dependent Genes during Neural Progenitor Differentiation. Representative scheme of the oxidation of TAF10 by LOXL2 after stem cell differentiation. The activity of LOXL2 leads to the degradation of TAF10 and the repression of pluripotency genes expression (Iturbide et al. 2014).

1.3.2. LOXL2 a new epigenetic writer

Interestingly, previous works from our group described a role for LOXL2 as an epigenetic eraser (Herranz et al. 2016; Millanes-

Romero et al. 2013). Indeed, LOX family members were shown to interact with histones (Kagan et al. 1983; Giampuzzi, Oleggini, and Di Donato 2003). For example, LOX protein has been reported to interact with histone H1 and its activity generates mitotic abnormalities.

Hence, LOXL2 specifically, deaminates trimethylated lysine 4 in histone H3 (H3K4m3) (Herranz et al. 2016) (Fig.i34). This reaction was described in the promoter of the cdh1 gene, collaborating with the SNAI1 transcription factor during the onset of the EMT. The down-regulation of H3K4me3 in cdh1 gene promoter contributes to the down-regulation of CDH1 protein. Indeed, as H3K4me3 is an active mark and its presence in the promoter stimulates the transcription of cdh1 gene, LOXL2 is considered a repressor enzyme that causes the deposition of an aldehyde group in the histone tail, the oxidized histone H3 (H3K4ox). Since, LOXL2, the eraser of H3K4me3 becomes the writer of H3K4ox.
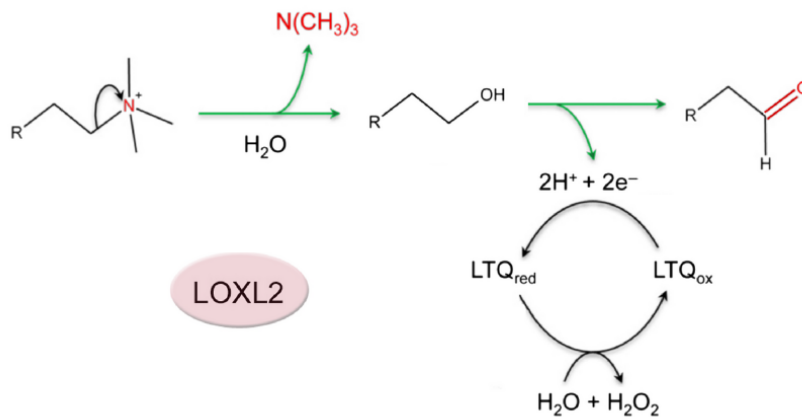
Figure.i34. LOXL2 oxidizes H3K4me3. Schematic diagram of the reaction of LOXL2 in the deamination of H3K4me3. Firstly, the catalytic activity of LOXL2 removes the amino group on the lyisine promoting the intermediate alcoholic form that is rapidly oxidized by the LTQ cofactor of LOXL2 generating the aldehyde group (Adapted from (Herranz et al. 2016)).

This deamination of histone 3 by LOXL2 has been described as crucial in the regulation of heterochromatin transcription during EMT (Millanes-Romero et al. 2013) (Fig.i35). LOXL2, in collaboration with SNAI1 deaminates H3K4me3 in the pericentromeric regions leading to a down-regulation of major satellite transcripts, a particular ncRNAs from the repetitive sequences in the pericentromeric regions of chromosomes. The down-regulation of the major satellites leads to a release of HP1α from the pericentromeric regions. The release of HP1α is necessary for chromatin reorganization. Thereby, during an EMT program, LOXL2 is necessary to induce global chromatin changes to allow a correct transition between the two phenotypic states. Subsequently, the activity of LOXL2 down-regulating the major satellite transcripts causes the release of HP1α allowing the chromatin reorganization necessary for the complete EMT.
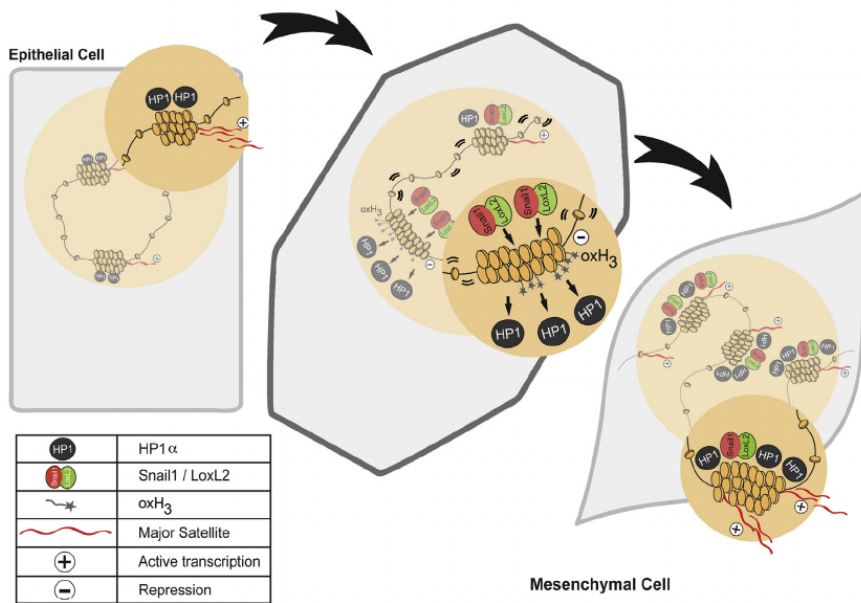
Figure.i35. LOXL2 activity is necessary for HP1α release to undergo EMT.
Upon TGF-β induction, SNAI1 and LOXL2 are rapidly upregulated and recruited to the pericentromeric regions oxidizing H3 and repressing major satellite transcription. This causes the release of HP1α from heterochromatin allowing chromatin reorganization and acquisition of mesenchymal traits (Millanes-Romero et al. 2013).

LOXL2 has been reported to be particularly enriched in breast cancer tumors (Barker, Cox, and Erler 2012; Ahn et al. 2013), and its presence correlated with metastasis and decreased survival in patients (Barker et al. 2011). Strikingly, upregulation of LOXL2 in various breast tumor cells has been shown to promote their invasiveness in vitro and in vivo (Moon et al. 2013; Hollosi et al. 2009). Thus, in the subgroup of triple-negative breast cancers (TNBC), there are evidences of increased levels of LOXL2 expression (Ahn et al. 2013; Y. Wang et al. 2016). For that reason,

we used breast cancer as a model to study the role of LOXL2 in breast cancer cells.

Accordingly, in this project we define the LOXL2 activity on the histone tail as a new epigenetic regulator. The deamination H3K4me3 opens the door to a new epigenetic mark, the H3K4ox. High levels of LOXL2 and H3K4ox in TNBC unveil the existence of an active molecular mechanism that ensures the compaction of heterochromatin regions to actively avoid the DDR pathway, which gives these cancer cells an evolutionary advantage despite the accumulation of DNA lesions.

2. METHODS

2.1. Laboratory procedures and materials

2.1.1. Cell Lines, Transfections, and Infections

All cell lines (HEK293T, basal breast cancer MDA-MB-231, luminal A T-47D, MCF-7, and luminal B BT-474) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (Invitrogen) at 37ºC in 5% $CO2$. For lentiviral infections to knock-down LOXL2, HEK293T cells were used to produce lentiviral particles. Cells were grown to 70% confluency and then transfected by drop-wise addition of a mixture of NaCl, DNA composed of 50% pLKO-shCT/shLOXL2, 10% pCMV-VSVG, 30% pMDLg/pRRE, 10% pRSV rev, and polyethylenimine polymer (Polysciences Inc), which was pre-incubated for 15 min at room temperature. The transfection medium was replaced with fresh medium after 24 hour, and the viral particles were concentrated using Lenti-X Concentrator product (Clontech). MDA-MB-231 cells were infected using the concentrated viral particles (Millanes-Romero et al. 2013). For retroviral infections, HEK293 gag-pol cells were used to produce retroviral particles. Cells were transfected as described for the HEK293T cells with a DNA mixture comprising DNA (2.5 µg of pCMV-VSV-G and 7.5 µg of pMSCV, pMSCV-LOXL2 wt-FLAG or pMSCV-LOXL2 mutFLAG ires GFP vectors) and polyethylenimine polymer (Polysciences Inc) that were pre-incubated for 15 min at room

temperature. The transfection medium was replaced with fresh medium after 24 hours, and the viral particles were concentrated using Retro-X Concentrator product (Clontech). MDA-MB-231 cells were infected using the concentrated viral particles. For LOXL2-Flag overexpression assays, MCF-7 cells were seeded for 24 hours and transfected with 10 µg of pcDNA3-hLOXL2-Flag vector using polyethylenimine polymer. For lentiviral infections to express H1-GFP, HEK293T cells were used to produce lentiviral particles. Cells were grown to 70% confluency and then transfected as explained with a mixture of NaCl, DNA composed of 50% FUGW-H1-empty vector/FUGW-H1-GFP, 10% pCMV-VSVG, 30% pMDLg/pRRE and 10% pRSV rev, and polyethylenimine polymer (Polysciences Inc), which was pre-incubated for 15 min at room temperature. The transfection medium was replaced with fresh medium after 24 hours and the viral particles were concentrated using Lenti-X Concentrator product (Clontech). MDA-MB-231 cells were infected using the concentrated viral particles.

2.1.2. Cell Extracts

To obtain nuclear fractions of LOXL2-Flag–transfected MCF-7 cells and HEK293T cells, cells were lysed in soft-lysis buffer (50 mM Tris, 2 mM EDTA, 0.1% NP-40, 10% glycerol, supplemented with protease and phosphatase inhibitors) for 5 min on ice. Samples were centrifuged at 3,000 rpm for 15 min, and the supernatant was discarded. The nuclear pellet was lysed in High-salt lysis buffer (20

mM HEPES pH 7.4, 350 mM NaCl, 1 mM MgCl2, 0.5% Triton X-100, 10% glycerol, supplemented with protease and phosphatase inhibitors) for 30 min at 4°C, and samples were centrifuged at 13,000 rpm for 10 min. Supernatant NaCl concentration was reduced to 300 mM NaCl with balanced buffer (20 mM HEPES pH 7.4, 1 mM MgCl2, 10 mM KCl). Cells extracts from MDA-MB-231, MCF-7, T-47D, and BT-474 cell lines were obtained with SDS lysis buffer (2% SDS, 50 mM Tris-HCl, 10% glycerol). Cell extracts of PDXs samples were obtained with SDS lysis buffer (2% SDS, 50 mM Tris-HCl, 10% glycerol). Proteins were separated by SDS-polyacrylamide gel electrophoresis gel and analyzed with the indicated antibodies.

2.1.3. Antibodies

The following antibodies were used: anti-FLAG (F7425, Sigma), anti-LOXL2 (NP1-32954, Novus), anti-H3K4me3 (07-473, EMD Millipore), anti-phospho-histone H2A.X (Ser139p) clone JBW301 (05-636, EMD Millipore), anti-GFP (ab6556, Abcam), anti-53BP1 (NB100-904, Novus Biologicals), anti-Phospho-chk1 (S317) (A300-163A, Bethyl), anti-Chk2 clone 7 (05-649, EMD Millipore), anti-cleaved caspase 3 (Asp175) (9661, Cell Signaling) and anti-histone H3 (ab1791, Abcam). An anti-H3K4ox antibody was developed using modified peptides coupled to Keyhole limpet hemocyanin (KLH).

2.1.4. Recombinant LOXL2 Purification

LOXL2-Flag recombinant proteins (wild-type and mutant) were purified from Sf9 insect cells (Herranz et al., 2016). Briefly, LOXL2-encoding baculovirus were amplified, and the proteins were produced in Sf9 cells according to standard procedures. Cell lysis was performed as previously described (M. Wu et al. 2008). Cell extracts were incubated with Flag M2 beads for 4 hour at 4ºC and washed 4× with Washing buffer (20 mM HEPES (pH 7.4), 1 mM MgCl2, 300 mM NaCl, 10 mM KCl, 10% glycerol, and 0.2% Triton X-100). Recombinant proteins were eluted with the Flag peptide (1 µg/µL) for 1 hour at 4ºC.

2.1.5. Dot Blot Assay

For dot blot assays, 1 µg of each peptide (in 10 µL of sample) were applied under low vacuum to a pre-wetted nitrocellulose membrane (Amersham Protran 0.45 nitrocellulose, GE Healthcare) using a dot blot apparatus (HYBRI-DOT Manifold; Life Technologies). The entire blot was blocked in 15 mL of 5% non-fat dry milk and 0.1% Tween-20 Tris-buffered saline (TBS) for 1 hour at room temperature and then probed with the indicated antibodies.

2.1.6. ChIP-seq and ChIP-qPCR experiments

ChIP experiments were performed similar as described (Herranz et al. 2016). Cells were crosslinked in 1% formaldehyde for 10 min at

37ºC. Crosslinking was stopped by adding glycine to a final concentration of 0.125 M for 2 min at room temperature. Cell monolayers were scraped in cold soft-lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.1% NP-40, 10% glycerol). Cell extracts were centrifuged at 3000rpm for 15 min.. Nuclei pellets were lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0) and incubated 20 min on ice. Nuclear extracts were centrifuged at 13000rpm for 10' and pellet and supernatants were kept. Supernatants were sonicated to generate 200- to 1,500-bp DNA fragments. Protein was quantified. For immunoprecipitation, supernatants were diluted 1:10 with dilution buffer, and precleared for 3 hours rotating at 4ºC with agarose beads (Diagenode #C03020003) and irrelevant IgGs. Samples were centrifuged 5 min at 2000rpm. Beads were discarded. Samples were incubated with rotation overnight at 4ºC with primary antibody or irrelevant IgGs. Samples were incubated with BSA-blocked beads for 3 hours rotating at 4ºC. Washing protocol was performed by column purification (Mobitec #M1002S). Samples were washed 3× with Low salt buffer (20mM Tris pH 8.0, 150mM NaCl, 2mM EDTA, 0,1% SDS, 1% Triton X-100), 3× with High salt buffer (20mM Tris pH 8.0, 500mM NaCl, 2mM EDTA, 0,1% SDS, 1% Triton X-100) and 2× with LiCl buffer (10mM Tris pH 8.0, 1mM EDTA, 1% NP-40, 1% NaDOC, 250mM LiCl). Samples were then treated with elution buffer (100 mM Na2CO3 and 1% SDS) for 1 hour at 37 ºC. After centrifugation for 3 min at 2000rpm beads were discarded. Eluted samples incubated at 65ºC overnight after addition of NaCl to a final concentration of 200 mM, to reverse formaldehyde

crosslinking. After proteinase K treatment for 1 hour at 55ºC, DNA was purified with MinElute PCR purification kit from Qiagen and eluted in Milli-Q water. Genomic regions were detected by quantitative PCR SYBR green staining (Qiagen), and the ChIP results were quantified relative to the input amount and the amount of H3 immunoprecipitated in each condition.

2.1.7. ATAC-seq and ATAC-qPCR experiments.

ATAC experiments were performed as described (Buenrostro, Wu, Chang, et al. 2015). Cells were harvested and treated with transposase Tn5 (Nextera DNA Library Preparation Kit, Illumina). DNA was first purified using MinElute PCR purification kit (Qiagen), samples were amplified by PCR using NEBNext High-Fidelity 2x PCR Master Mix (New Englands Biolabs), and DNA was again purified with the MinElute PCR purification kit. Finally, qPCR with the same primers as in the ChIP experiments was performed on a 1/50 dilution of the eluted DNA.

2.1.8. Primer list.

Gene	Forward / Reverse
prHPRT	ATTCACGCGATGACTGGA AGGCTCACTAGGTAGCCGTG
prRNAPolII	CTGAGTCCGGATGAACTGGT ACCCATAAGCAGCGAGAAAG
Hit1_chr14	ATAAGCTTTTTGATGTGCTGCTG GAGCTGCTAGCATTCCTTCTAA
Hit2_chr5	TAACTCATTTATGAGGCCAACGTC CTTGTGCATATTGAACCAGCCT
Hit4_chr2	AGCTCTGTAAGAACTAAGATTGGGCT TTCTATCTTCACGGTTCTCCAAGA
Hit5_chr17	TAAGAGAGCCTTGCATCCCA AGGCCAGCATCATCCTGATA
Hit6_chr4	AGGGGATATCACTGCCGATC TTATTGCCACAATTTCAGAGCC
Hit7_chr6	GCCTTTGACAAACTTCAACAATG TAAATAGGAGTGGTGAAGGAGGG
Hit8_chr5	TGTATTTCTGTGGGATCAGTTGG GCAGAACTGAAGGAGATAGAGACACA
Hit9_chr13	GTGATTATATACCACCATTTGCCCT GAAAGGCATTGGTAGCTTGATG
prSMIM5	CAAGGGAAATTGTCCAGACTTC AGTAGCTGGGACTACAGGCG
LOXL2	ACTGACTGCAAGCACACGGA TCAGGTTCTCTATCTGGTTGATCAA
Pumilio	CGGTCGTCCTGAGGATAAAA CGTACGTGAGGCGTGAGTAA

Table.2. Human primers used for mRNA, ChIP-qPCR and ATAC-qPCR analysis. The sequences of the different human primers used in this study are listed. All of them are shown in 5' to 3' direction. The eleven first primers amplify genomic DNA and were used in the ChIP-qPCR and in the ATAC-qPCR while the two shadowed ones were used in the mRNA analysis.

2.1.9. Cell Cycle Analysis

MDA-MB-231–infected cells under selection were first synchronized through the double thymidine block protocol. Cells were seeded to 50% confluency and then incubated for 14 hours with complete growth medium supplemented with 2 mM thymidine. Cells were washed 2× with PBS and released by a 9-hours incubation with complete medium growth. Cells were washed again 2× with PBS and subjected to a second 14-hours incubation with 2 mM thymidine. Finally, cells were released with the complete growth medium until harvesting at the designed time points. Cells were harvested and fixed with 100% cold ethanol. Two days later, fixed cells were stained with propidium iodide (PI) staining and analyzed by flow cytometry using BD FACSCalibur (Becton Dickinson). Results were analyzed using BD CellQuest Pro software.

2.1.10. Cell Irradiation

MDA-MB-231–infected cells under selection were seeded for 24 hours after selection. Cells were then irradiated using γ-irradiation until 0.5 Gy was achieved. Cells were fixed at designed time points.

2.1.11. Non-Replicative Cell Experiment

MDA-MB-231 cells were seeded in coverslips and maintained during all the experiment in Dulbecco's modified Eagle's medium (Invitrogen) with 0.5% fetal bovine serum (Invitrogen) at 37ºC in 5% CO2. After 24 hours, cells were infected with lentiviral particles for LOXL2 knock-down. After 96 hours under selection, cells were fixed.

2.1.12. Rescue Experiments and Sorting

MDA-MB-231–infected cells under selection were seeded for 24 hours after selection. Cells were then re-infected with retroviral particles for LOXL2-FLAG or lentiviral particles for H1 expression. After 24 hours, cells were fixed. GFP-positive cells were sorted using BD INFLUX (Becton Dickinson) and analyzed using BD FACS SORTWARE 1.0.0.650.

2.1.13. Immunofluorescence, Image Acquisition, and Analysis

Cells were fixed with 4% PFA for 15 min at room temperature, blocked for 1 hr with 1% PBS-BSA, incubated at room temperature for 2 hours with primary antibody, washed 3x with PBS, and then incubated for 1 hour at RT with the secondary antibody. Cells were washed again 3x with PBS, incubated for 5 min with DAPI (0.25 mg/mL) for cell nuclei staining, and then mounted with fluoromount. Fluorescence images corresponding to DAPI,

γH2A.X, 53BP1 and H3K4ox were acquired in a Leica TCS SPE microscope using a Leica DFC300 FX camera and the Leica IM50 software. Analyses of intensity and number of dots were performed with the ImageJ software by first defining the cell nuclei with DAPI staining. Max projection of the confocal images was performed. To define the expression of γH2A.X and 53BP1 per cell, the average intensity of pixels in the reference channel (Alexa 488, Alexa 555) and the number of dots within the defined nuclear region was measured.

2.1.14. Breast Cancer Patient-Derived Xenografts (BC-PDXs)

BC-PDXs were generated as previously described (Morancho et al. 2016). Briefly, six- to eight-week-old female NOD.CB17-Prkdc<scid>J (NOD/SCID) mice were purchased from Charles River Laboratories (Paris, France). Mice were maintained and treated according to institutional guidelines of Vall d'Hebron University Hospital Care and Use Committee. Fragments of patient samples were implanted into the fat pad of the mice, and 17 ß-estradiol (1 μM) (Sigma) was added to drinking water. The breast tumor samples used in this study were either from a surgical resection (PDX-71, PDX-251, PDX-8, PDX-345 and PDX-385) or a cutaneous metastasis biopsy (PDX-118) obtained at Vall d'Hebron University Hospital following the institutional guidelines. Written informed consent for the performance of tumor molecular studies was obtained from the patients who provided tissue. Histopathologic characteristics were confirmed by a pathologist.

PDX-71 is a luminal B breast tumor carrying a BRCA2 gene mutation c.3264dupT in exon11; PDX-251 and PDX118 are luminal B, HER2-positive breast tumors; and PDX-8, PDX-347, and PDX-385 are TNBC tumors.

To perform ATAC-qPCR protocols with these samples, tumoral cells were isolated from PDXs using the tumor dissociation kit (MACS, Miltenyi Biotech) to obtain a single cells suspension.

Representative westerns blots used to detect LOXL2 and H3K4ox expression levels in these samples were digitally analyzed using the software package Photoshop CS4. Integrated densities for each band were determined for the background and for each protein of interest and its corresponding loading control (Tubulin for LOXL2 and total H3 for H3K4ox). The background was subtracted for each specific band, and the ratio of band intensity of LOXL2 and H3K4ox of the corresponding loading control was used as the relative protein expression level.

2.2. Bioinformatic processing

2.2.1. ChIP-seq analysis

Peaks of H3K4ox were called from sequence reads detected through ChIP-seq using the MACS2 tool (Zhang et al., 2008) . The chromatin states files for Hpeg2 and HMEC cells were computed by the ENCODE project using the ChromHMM tool (Ernst and Kellis, 2012) from https://genome.ucsc.edu/cgi-

bin/hgFileUi?db=hg19&g=wgEncodeBroadHmm. The statistical overrepresentation of H3K4ox peaks detected by ChIP-seq was assessed, across several chromatin states from these two cell lines: heterochromatin, repressed, insulator, strong enhancer (sum of states 4 and 5), weak enhancer (sum of states 6 and 7), promoter (sum of states 1 and 2), and poised promoter. The contingency table of the Fisher's test carried out to this end contained the number of nucleotides within: peaks, chromatin states, intersections thereof, and the remaining portion of the genome (computed as the difference from the effective genome size for ChIP-seq peaks calling). The same procedure was applied to detect the overrepresentation of oxH3 peaks in lamin-associated domains of chromatin, obtained from (Guelen et al., 2008).

2.2.2. ATAC-seq analysis

Reads produced by ATAC sequencing of two control (shControl) replicates and two LOXL2 knock-down sequencing replicates (shLOXL2) were aligned to the hg19 build of the reference human genome using Bowtie2 (Langmead et al., 2009) with default parameters for pair-end sequencing. ATAC peaks were then called combining aligned reads of both replicates of the control and the knock-down using the MACS2. No FDR restrictions were imposed on the ATAC peak calling, to allow for FDR threshold selection further downstream in the analysis.

2.2.3. RNA-Seq Analysis

Reads produced by RNA sequencing of the same two control (shControl) and two LOXL2 knock-down (shLOXL2) replicates as above were aligned to the hg19 build of the reference human transcriptome using TopHat2 (Kim et al., 2013) with default parameters for pair-end sequencing. Aligned reads were then analyzed using a standard Cufflinks (Trapnell et al., 2012) pipeline to detected differentially expressed genes between the two conditions (LOXL2 knock-down and shControl).

2.2.4. Replicates Correlation

The read count (coverage) at each position of the hg19 human reference genome was computed for each replicate of the H3K4ox ChIPseq, and the shControl and shLOXL2 ATAC sequencing using the bedtools (Quinlan and Hall, 2010) genomecov capability. Genomic positions with zero read counts were filtered out. Replicates files of each experiment (or control) were merged to produce a single file aligned by genomic position, and the corresponding Pearson's correlation coefficient of read counts were computed. For the graphical representation of the correlation, 100,000 genomic positions were randomly selected.

2.2.5. Analysis of ATAC Peaks Overlapping H3K4ox Peaks

First, we intersect all significant ($p < 10–5$) ATAC peaks (shLOXL2 versus shControl) and H3K4ox peaks with the bedtools (Quinlan and Hall, 2010) intersect program. Based on this intersection, ATAC peaks were classified as overlapping (if they intersected an H3K4ox peak) or orphan (if not). Only intersections involving more than 95% of the sequence of ATAC peaks were considered. shControl and shLOXL2 read counts over all genomic positions (see above) were intersected with both overlapping and orphan peaks. Read counts over genomic positions of control and experiment replicates were averaged. To carry out the heatmap representation, peak sequences (overlapping or orphan) were aligned by their summits. For the linear representation, the average experimental read counts at each downstream and upstream position were summed, and the same was done for the average control read counts at each position. Position-wise sums were then divided by the read count sum value obtained for the summit of control read counts, thus making all sums relative to the maximum control value.

2.2.6. Integrated Analysis of H3K4ox and ATAC Peaks and Differentially Expressed Genes

We selected the differentially expressed genes detected through the RNAseq analysis of shControl and shLOXL2 cells that were close (upstream or downstream) overlapping ATAC peaks. We employed two different distance thresholds to detect close differentially expressed genes: 0.5 Mb and 1 Mb.

3. OBJECTIVES

Deamination of trimethylated lysine 4 in histone 3 by LOXL2 has been described as a key step in EMT and in cancer progression. However, the relevance of the released oxidation in histone 3, H3K4ox, still remains elucidated.

The general objective of this thesis is thus to describe the role and relevance of H3K4ox mark in the histone tail. To this aim we focused on:

I. Generation and validation of an antibody against H3K4ox

II. Characterization of H3K4ox in the breast cancer model

III. Finding biological relevance of H3K4ox and LOXL2 in TNBC cells and PDXs.

4. RESULTS

4.1.H3K4ox is a new epigenetic mark

LOXL2 reaction on H3K4me3 generates an aldehyde group highly reactive (Herranz et al. 2016). The described reaction involves a nucleophilic attack by a water-derived OH^- to the $C\varepsilon$ of the lysine with the release of the $N(CH_3)_3$. The generated alcohol is rapidly oxidized by the internal redox cofactor lysine-tyrosilquinone (LTQ) driving the aldehyde group release in the histone tail. The LTQ is reoxidized with the consequent release of H_2O_2. The high reactivity of the aldehyde group does not allow the generation of an antibody against this chemical group. Hence, we raised a modification-specific antibody that detects the intermediate alcohol of LOXL2 as readout of the oxidized H3K4. This antibody was produced using a peptide (Fig.r1A) containing the artificial amino acid 6-hydroxinorleucine.

We demonstrated the specificity of the antibody using dilution series of peptides with the histone tail sequences and different modifications, verifying that the H3K4ox antibody only recognizes specifically the H3K4ox peptide but not an unmodified peptide of the H3 tail neither modified peptides with a methylation in lysine 4 or 9 (H3K4me3 and H3K9me3 respectively) (Fig.r1B). Moreover, its specificity was further demonstrated by Western Blot, immunofluorescence and ChIP experiments (Fig.r1C-1H). Then, we performed an in vitro reaction using purified nucleosomes and

recombinant LOXL2 proteins. Purified nucleosomes were obtained from 293T cells after a MNase treatment. Recombinant proteins were obtained from Sf9 insect cells using LOXL2-encoding baculovirus. The mutant LOXL2 contains two point mutations in the positions 626 and 628 were two histidine residues were mutated to glutamine. These residues are predicted as Cu(II) binding sites and are important for the catalytic activity of all LOX family members (Herranz et al. 2016). Accordingly, H3K4ox was enriched, with a corresponding reduction in H3K4me3 levels, in the LOXL2 wild-type (LOXL2) compared to the catalytically inactive mutant (LOXL2mut) (Fig.r1C). In addition, transient transfection of LOXL2 flag-tagged in breast cancer cells MCF-7, a cell line with an epithelial phenotype and low basal levels of LOXL2 (D. L. Holliday and Speirs 2011; Ahn et al. 2013), increased the levels of H3K4ox compared to the empty-vector (Ø) transfected cells (Fig.r1D). Also, LOXL2 was depleted from a mesenchymal-like breast cancer MDA-MB-231 cells with high expression of LOXL2 (D. L. Holliday and Speirs 2011; Ahn et al. 2013) using a short-harpin RNA (shRNA) that interferes with the mRNA of LOXL2 (Herranz et al. 2016). The down-regulation of LOXL2 in MDA-MB-231 cells leads to a reduction in H3K4ox levels compared to cells infected with an irrelevant shRNA (shControl) by Western blot analysis (Fig.r1E) and in ChIP-qPCR experiments on the previously described target E-cadherin gene promoter (Herranz et al. 2016) (Fig.r1F). Finally, H3K4ox showed a nuclear location by immunofluorescence experiments both in MDA-MB-231 cells

(Fig.r2A) and in mouse embryonic fibroblast (MEFs) cells (Fig.r2B).
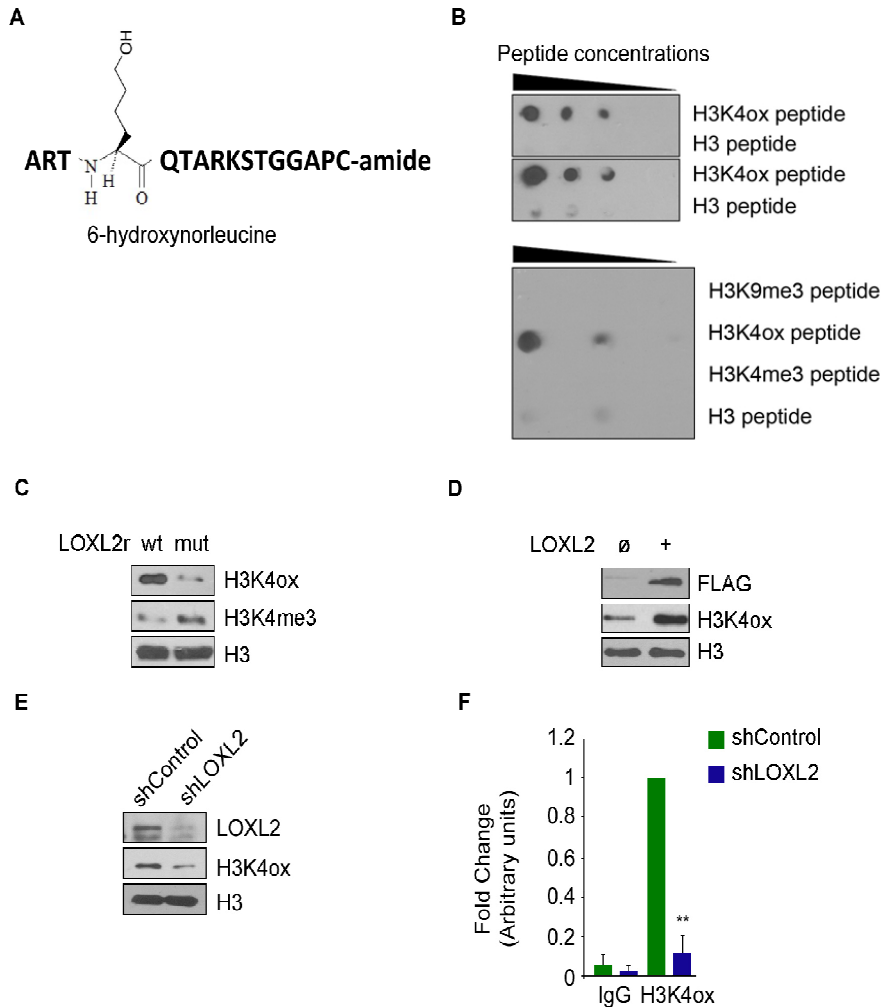
Figure.r1 Quality Control of the Anti-H3K4ox antibody. (A) Diagram of the peptide used to develop the anti-H3K4ox antibody with the artificial aminoacid a6-hydroxynorleucine. (B) Western blot using the anti-H3K4ox antibody in two replicates of dot blots of dilution series of oxidized histone H3 peptide or unmodified H3 peptide (upper panel) and in a representative dot blot of dilution series of H3K9me3, H3K4ox, H3K4me3, and H3 peptide (lower panel). (C) Recombinant LOXL2 wild-type (wt) or a catalytically inactive LOXL2 (mut) purified in baculovirus were incubated 4 hr at 4ºC with nucleosomes, and the

formation of H3K4ox and the decrease of H3K4me3 were detected with the indicated antibodies. (D) Western blot with the indicated antibodies on lysates of MCF7 cells transfected with an empty vector (ø) or with LOXL2. (E) Western blot for LOXL2, H3K4ox, and total H3 in MDA-MB-231 cells infected with short hairpin RNA as a control (shControl) or specific for LOXL2 (shLOXL2). (F) Anti-H3K4ox Chip-qPCR for cdh1 promoter in MDA-MB-231 cells infected with shControl (grey bar) or shLOXL2 (black bar). Data of qPCR amplification were normalized to the input and to total H3 and expressed as the fold-change relative to the data obtained in shControl conditions, which were set as 1.
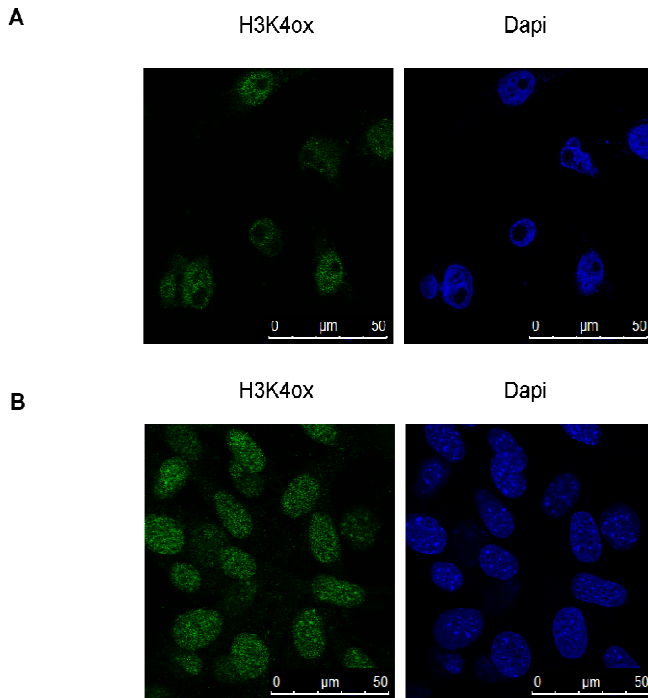
Figure.r2 Nuclear distribution of the H3K4ox antibody. (A-B) Confocal microscopy of MDA-MB-231 and MEFs cells, respectively, using the anti-H3K4ox antibody (green). Nuclei were staining with DAPI.

4.2. H3K4ox is enriched in TNBC breast cancer cell

Since LOXL2 expression correlates with cancer (Barker, Cox, and Erler 2012) and particularly with breast cancer (Cano, Santamaría, and Moreno-Bueno 2012), we checked the levels of LOXL2 and H3K4ox in several breast cancer cell lines: luminal A T-47D and MCF-7 cells lines (ER+/HER2-/PR+/-), luminal B BT-474 cell line (ER+/HER2+/PR+/-) and the basal triple negative breast cancer cell line MDA-MB-231 (ER-/HER2-/PR- e.g. TNBC) (D. L. Holliday and Speirs 2011). We observed high levels of H3K4ox in the TNBC cells by Western blot analysis (Fig.r3A) and this correlated with higher amount of LOXL2 both at protein (Fig.r3A) and mRNA expression (Fig.r3B).
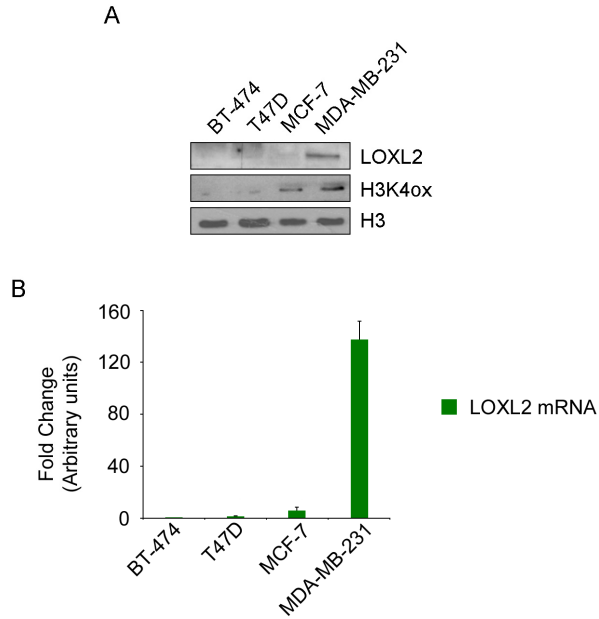
Figure.r3 H3K4ox is enriched in TNBC breast cancer cell. (A) Western blot for the indicated antibodies in a panel of breast cancer cell lines. (B) LOXL2 mRNA levels for the indicated breast cancer analyzed by RT-qPCR. Data of qPCR amplification were normalized to the Pumilio housekeeping gene and expressed as the fold-change relative to the data obtained in BT-474 cells, which was set as 1.

4.3. H3K4ox localizes in heterochromatin in TNBC MDA-MB-231 cells

In order to explore the in vivo function of H3K4ox in TNBC breast cancer cells, ChIP-seq experiment was performed in human breast cancer MDA-MB-231 cell lines, using the anti-H3K4ox antibody to determine the genomic distribution of this H3 modification. Peaks called using Model-based Analysis for ChIP-seq (MACS) (Yong Zhang et al. 2008) showed low differences in H3K4ox distribution

in two sequencing replicates with a 0,997 Pearson correlation coefficient (Fig.r4A). MACS is an algorithm that capture local biases in the genome improving the robustness and specificity of the prediction. Then, using the ChromHMM tool (Ernst and Kellis 2012), chromatin was classified to the different states that have been computed by the ENCODE project. ChromHMM is a software based on a Hidden Markov Model that allows the characterization of chromatin states. It integrates multiple chromatin datasets in order to discover de novo the major re-ocurring combinatorial and spatial patterns of a mark. Hence, statistical overrepresentation of the H3K4ox peaks through different chromatin states: promoter, weak and strong enhancer, insulator, repressed chromatin and heterochromatin. The contingency table of the Fisher's test carried out for this contained the number of nucleotides in: peaks, chromatin states, intersections thereof, and the remaining portion of the genome (computed as the difference from the effective genome size for ChIP-seq peaks calling) (Fig.r4B). The same procedure was applied to detect the overrepresentation of H3K4ox peaks in already published lamin-associated domains (LADs) (Guelen et al. 2008). Fisher's test uncovered a significant overrepresentation of H3K4ox in heterochromatin and LADs (Fig.r4B). A large fraction of H3K4ox peaks from both replicates were located within heterochromatin regions and LADs. (Figr4C-D) We validated this H3K4ox enriched regions from the ChIP-seq by ChIP-qPCR assay in shControl and shLOXL2 cells (Fig.r5). The results showed a decrease in the H3K4ox enrichment in the selected regions when LOXL2 was knocked-down with no changes in an irrelevant

promoter. This distribution suggested a correlation between H3K4ox and chromatin compaction as heterochromatin characterizes to be highly compacted and H3K4ox, as observed, mainly concentrated in this region.
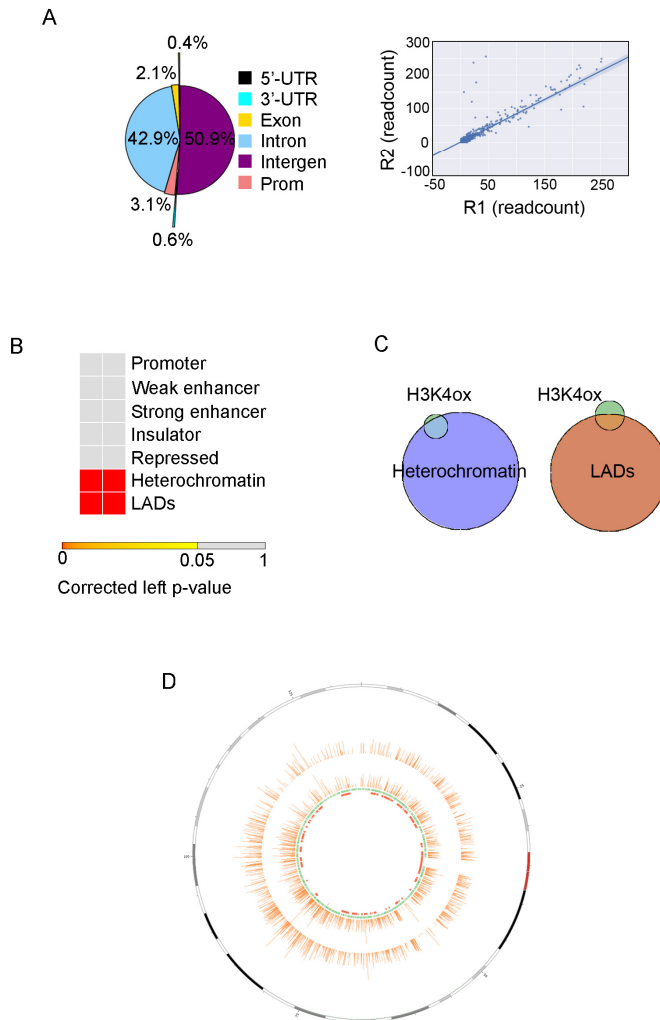
Figure.r4 H3K4ox localizes in heterochromatin in TNBC breast cancer MDA-MB-231 cells I. (A) Distribution of all H3K4ox ChIP-seq peaks in MDA-MB-231 cells, with the indicated percentages shown. Pearson correlation was used. (B) Contingency table of the Fisher's test showed the statistical overrepresentation of the H3K4ox peaks through different chromatin states. (C)

Venn diagrams representing the enrichment of H3K4ox peaks in heterochromatin and LADs. (D) Circus plot illustrating the location of H3K4ox (orange) peaks in both replicates across chromosome 12, with heterochromatic regions (green) and LADs (red) in the chromosome represented in the innermost tracks.
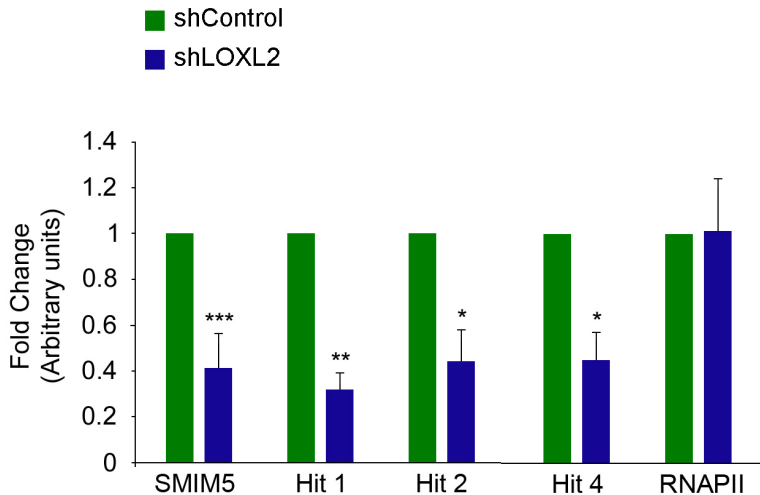
Figure.r5 H3K4ox localizes in heterochromatin in TNBC breast cancer MDA-MB-231 cells II. H3K4ox and LOXL2 Chip-qPCR validation for the selected hits in MDA-MB-231 cells infected with shControl or shLOXL2. Data of qPCR amplifications were normalized to the input and to total H3 for H3K4ox and expressed as a fold change relative to the data obtained in shControl conditions, which was set as 1. The RNA pol II promoter (RNAPII) was used as a negative control. *p<0.05, **p<0.01, ***p<0.001.

4.4. Chromatin conformation is altered in MDA-MB-231 cells in absence of LOXL2 and decreased levels of H3K4ox

As previously explained, heterochromatin characterize for being highly compacted compared with euchromatin. This difference in compaction, the striking distribution of H3K4ox in the heterochromatin and the removal of an active mark as H3K4me3 suggested an important role for this mark in chromatin structure.

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Hence, chromatin conformation was determined using the assay for transposase-accessible chromatin (ATAC) (Buenrostro, Wu, Chang, et al. 2015; Tsompana and Buck 2014a) followed by deep-sequencing (ATAC-seq). This technique exploits the ability of the prokaryotic transposes Tn5 to integrate into accessible (open) regions of chromatin. We assessed the conformation of chromatin in MDA-MB-231 cells knock-down for LOXL2 (shLOXL2) compared to shControl. The results showed an increased in accessibility at the H3K4ox-positive sites in shLOXL2 compared to shControl and minor differences in H3K4ox-negative regions (Fig.r6). Heatmaps show the ATAC signals in H3K4ox positive and negative regions from the ChIP-seq in shLOXL2 versus shControl. These results were validated by ATAC-qPCR (Fig.r7). The regions used in the ChIP-qPCR showed an increased accessibility in shLOXL2 compared to shControl and not in an irrelevant promoter. So, these data suggest that, in absence of LOXL2 and with a decreased H3K4ox levels, chromatin adopts a more open conformation suggesting a new putative role for LOXL2 in directly maintain a subset of closed chromatin regions throughout the oxidation of H3. Since the generation of the aldehyde in H3 results in a loss of the positive charge and the creation of a very reactive group could explain this particular chromatin conformation. Moreover, these changes in chromatin conformation with a transposes-increased accessibility and reduced levels of H3K4ox did not correlate with increase gene transcription. RNA-seq in shLOXL2 cells found 339 deregulated genes (FC, 2; q value < 0,05), of which 151 were up-regulated and 188 were down-regulated. Furthermore, the

transcriptional behavior of those genes close to the ATAC+/H3K4ox+ sites (at 0,5 Mb, 1 Mb, and 5 Mb) still did not reveal any preference, suggesting that chromatin accessibility and H3K4ox is not related to transcriptional repression but rather to chromatin conformation (Fig.r8).
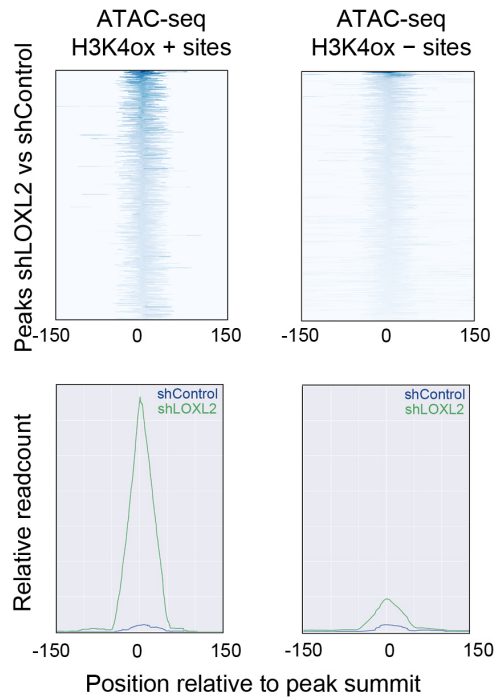
Figure.r6 Chromatin conformation is altered in absence of LOXL2 (I). Significant (p < 1 x10-5) ATAC peaks overlapping chromosomic regions (left panel graphs) within significant (p < 1 x10-5) H3K4ox ChIP peaks exhibit higher normalized read count than non-overlapping ATAC peaks (right panel graphs). Heatmaps at the top represent the difference of read count (between the knocked-down and control cell lines) at genomic positions covered by peaks. Peaks are stacked as the rows of the heatmap, whose columns are relative genomic positions, centered at peaks summits. Analogously, bottom panels represent the quotient of normalized read count in the knocked-down and control cell lines as a continuous line. The value of the line in each point is the sum of the read count at the corresponding relative position of the peak, resulting from centering all peaks at their summits. For the sake of comparison, right panel graphs represent only the top-ranking non-overlapping 339 peaks (equal to the number of overlapping peaks).
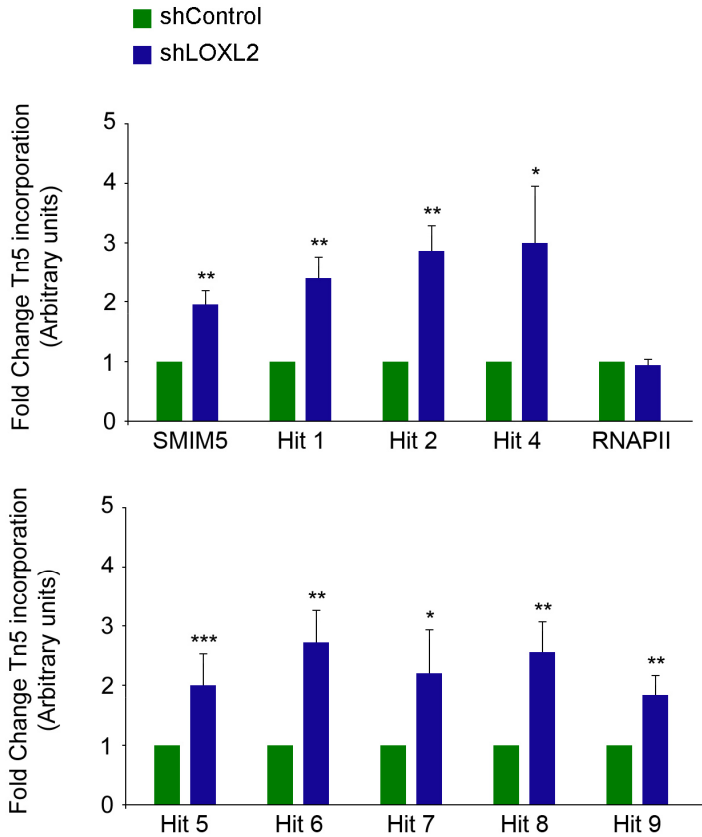
Figure.r7 Chromatin conformation is altered in absence of LOXL2 (II). Chromatin compaction was assessed in MDA-MB-231 infected with shControl or shLOXL2 by ATAC-qPCR. Chromatin was obtained from shControl and shLOXL2 cells and then was treated with the transposase Tn5 that will be incorporated only in regions with an open chromatin. qPCR amplification of the H3K4ox-selected hits were normalized to unchanging genomic region (HPRT) and expressed as the fold-change relative to the data obtained in shControl conditions, which were set as 1. RNAPII was used as a control. Error bars indicated standard deviation of at least three independent experiments. *p<0.05, **p<0.01, ***p<0,001.
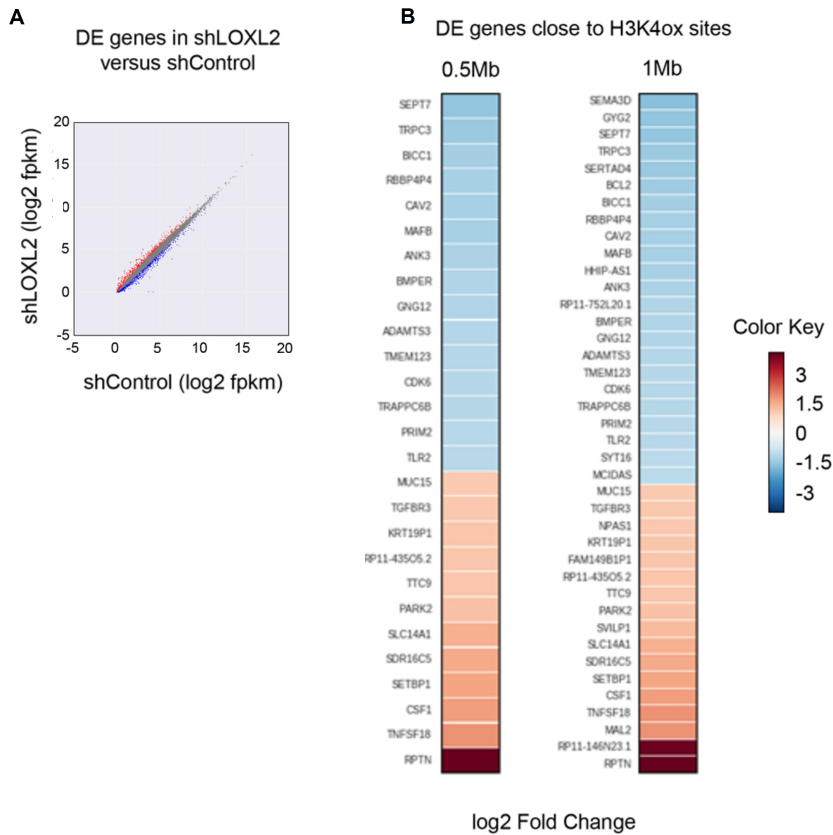
Figure.r8 H3K4ox is not related to transcriptional repression. (A) Differentially expressed genes in shLOXL2 compared with shControl (B) Heatmaps of the differentially expressed genes in MDA-MB-231 cells infected with the shLOXL2 compared to shCT. Each heatmaps contains those genes close to ATAC+/H3K4ox+ sites (at 0.5 Mb and 1 Mb). RNA-seq in shLOXL2 cells found 339 deregulated genes (FC, 2; q value < 0.05), of which 151 were up-regulated (blue colour) and 188 were down-regulated (red colour).

4.5. Increased accessibility in heterochromatin of MDA-MB-231 cells activates DDR

As described in the introduction, somatic mutations and DNA lesions in cancer tend to accumulate in heterochromatin (Schuster-Böckler and Lehner 2012; Supek and Lehner 2015), and since there is a clear relation between DDR and chromatin accessibility, we hypothesized whether there is any correlation between LOXL2 and H3K4ox in heterochromatin and DDR. For this purpose, staining for phosphorylated H2AX (ɣ-H2AX) and 53 binding protein (53BP1) were performed at 2 days after puromycin selection in MDA-MB-231 cells infected with shLOXL2 or shControl (Fig.r9). As previously seen, the recruitment of these two proteins is an early step in the DDR signaling after a DSB lesion. Interestingly, we observed a higher number of ɣ-H2AX and 53BP1 foci in shLOXL2 compared to shControl cells. These results suggested an increased DDR activation in the absence of LOXL2 and within an open chromatin context. In addition, we analyzed the cell cycle profile to measure the output of DDR activation and accordingly, the knocked-down cells, the ones with increased DDR and open chromatin, were arrested in G1/S. Fluorescence-activated cell sorting (FACS) analysis of asynchronous cells did not show significant differences in cell cycle progression (Fig.r10A). Then, in order to address the arrest, we performed a synchronization assay consisting in the induction of cellular arrest by blocking the replication machinery due to an excess of the nuclear base pair

thymidine. Hence, cells get arrested in the early moments of S phase (H. T. Ma and Poon 2011). Here, after synchronization with double thymidine blockage shLOXL2 cells were not able to progress throughout the S phase and were arrested in the early moments of the cell cycle (Fig.r10B).
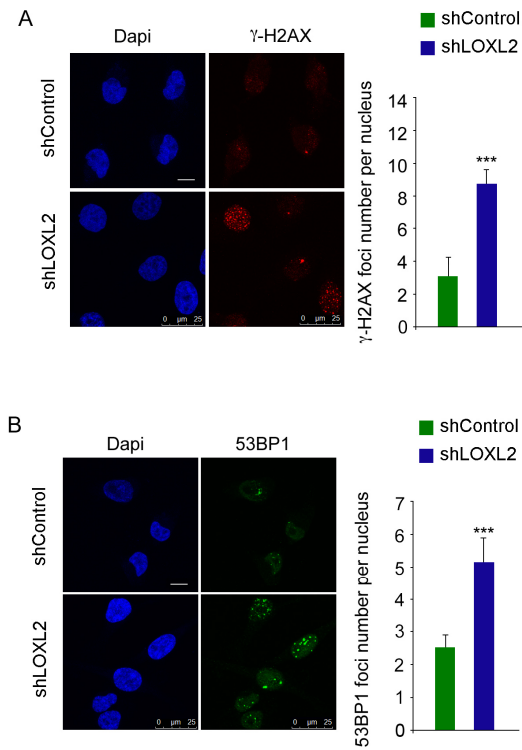
Figure.r9 MDA-MB-231 cells lacking LOXL2 showed increased DDR. (A) ɣ-H2AX staining and foci quantification is showed by immunofluorescence with and specific antibody for ɣ-H2AX. Graphs indicated changes in the number of the ɣ-H2AX foci in shControl and shLOXL2 MDA-MB-231 cells. (B) 53BP1 staining and foci quantification is showed by immunofluorescence with and specific antibody for 53BP1. Graphs indicated changes in the number of the 53BP1 foci in shControl and shLOXL2 conditions. Nuclei were staining with DAPI. ***p<0,001.
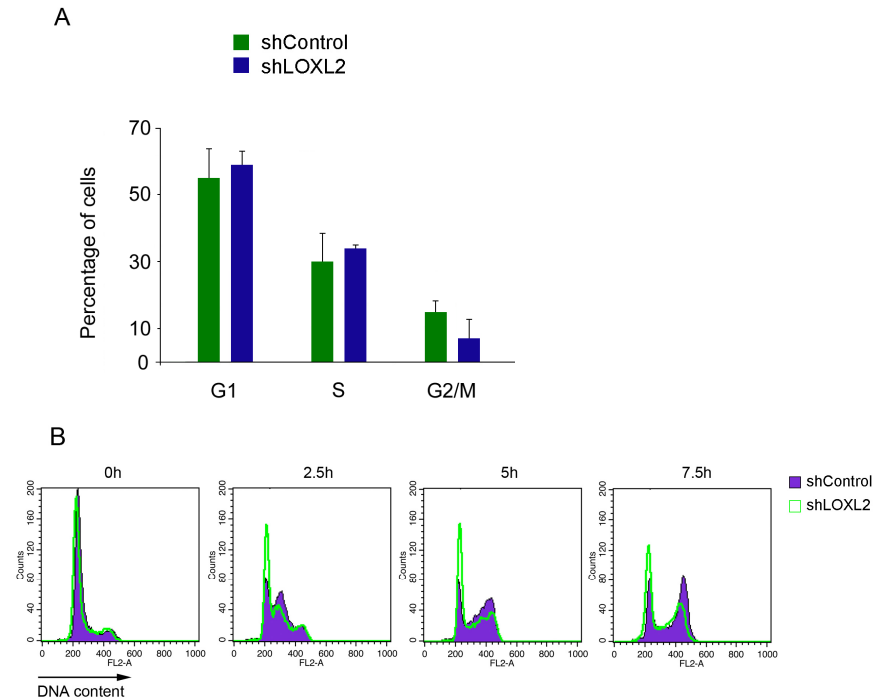
Figure.r10 Knocking-down LOXL2 arrest MDA-MB-231 cells in G1/S phase. (A) Quantification of three different experiments using asynchronous shControl and shLOXL2. (B) Cell cycle profile of shControl and shLOXL2 MDA-MB-231 cells at 0, 2.5, 5, and 7.5 hours upon release from a double thymidine block. Cells were analyzed by FACS upon propidium iodide staining.

These results suggest an activation of the G1/S checkpoint in shLOXL2 cells. Consistently, we observed the phosphorylation of both checkpoint kinase 2 (Chk2) (Sancar et al. 2004) and cell cycle checkpoint 1 (Chk1) (Zhao, Watkins, and Piwnica-Worms 2002) (Fig.r11A). Here, in order to elucidate if the increase in the ɣ-H2AX foci number was due to the entrance into the apoptosis pathway in shLOLX2 cells at this time point, we analyzed by Western Blot the presence of cleaved caspase-3. However, we discarded it, as we did not observe either in shControl or shLOXL2 cells (Fig.r11B). These results are in accordance with the idea that reduced amount of

H3K4ox and an open chromatin context in the absence of LOXL2 enhances the DDR. Despite the aberrant activation of DDR compared with shControl cells, shLOXL2 cells are more sensitive and are not able to survive, as shown by colony-formation (Fig. r11C).
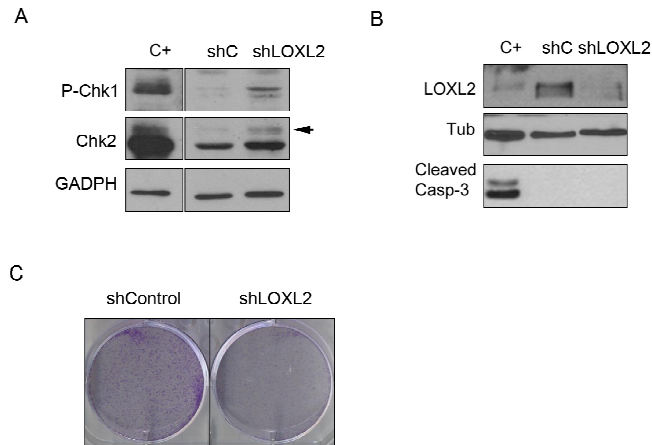
Figure.r11 In absence of LOXL2, MDA-MB-231 cells activate cell cycle checkpoints but not the apoptosis pathway. (A) Western blot analysis for the indicated cell cycle checkpoint proteins in shControl and shLOXL2 MDA-MB-231 total extracts. GAPDH was used as a loading control (B) Western blot analysis using shControl and shLOXL2 MDA-MB-231 total extracts to the indicated marker of apoptosis. Tubulin was used as a loading control. Activated fibroblasts were used as a positive control for the antibodies. (C) Colony-survival assay in shControl and shLOXL2 MDA-MB-231 cells.

Moreover, the damage observed may be generated the novo by altering the chromatin structure inducing problems in the replication of the DNA (Zeman and Cimprich 2013). Indeed, stalled DNA replication forks are a major source of endogenous DNA damage, particularly in cancer cells (Khurana and Oberdoerffer 2015). So, in order to discard replicative stress, we analyzed the number of ɣ-H2AX and 53BP1 foci in non-replicative cells. However, results

still showed enhanced activation of the DDR in non-replicative shLOXL2 cells compared with control ones (Fig.r12). Hence, we can discard replicative stress as the damage source in shLOXL2. Considering, our data suggests that in TNBC cells, changing the chromatin conformation to a more accessible state exposes the DNA damage and DNA lesions, allowing the DDR to be activated more efficiently than in TNBC cells with closed chromatin. Importantly, the TNBC cells in the open chromatin state are not able to repair and die after several days in culture.
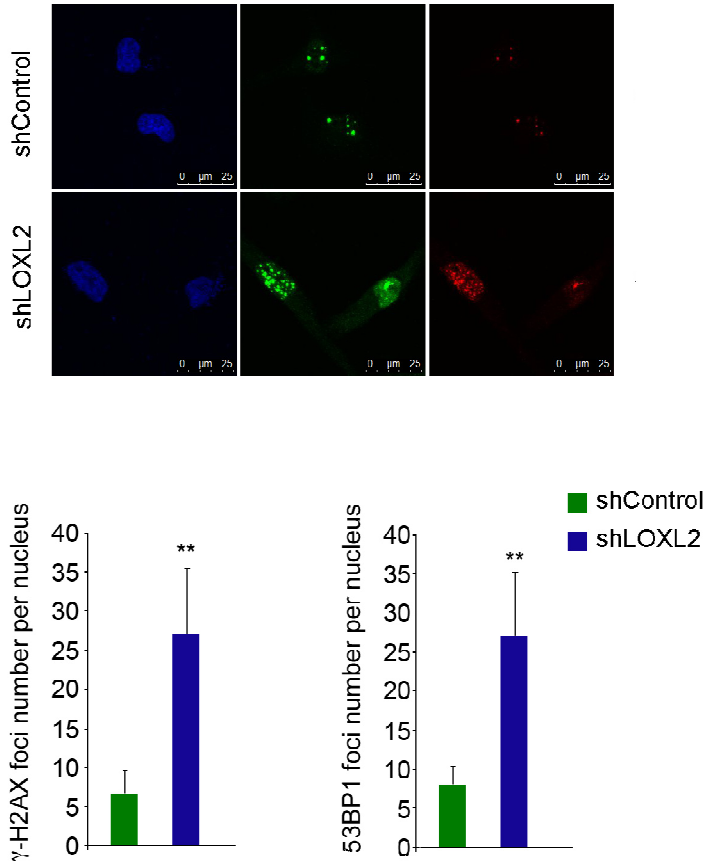
Figure.r12 Enhanced DDR in the absence of LOXL2 persists in non-replicative cells. ɣ-H2AX and 53BP1 staining and foci quantification are showed by immunofluorescence with the indicated antibody. Graphs indicated changes in the number of the ɣ-H2AX and 53BP1 foci in shControl and shLOXL2 MDA-MB-231 cells under non-replicative conditions. ** $p<0,01$.

4.6. Rescue experiments confirm the role of chromatin condensation by LOXL2 and H3K4ox in DDR activation.

Based in the above observations, we hypothesized that LOXL2 induces a structural change in heterochromatin through the oxidation of histone H3, leading to a more compacted chromatin and a less efficient DDR. To further confirm the role of the catalytic activity of LOXL2, a rescue experiment was performed. shLOXL2 cells were re-infected with the ectopic vector expressing LOXL2-ires-GFP or a version of LOXL2 catalytically inactive (Herranz et al. 2016) LOXL2mut-ires-GFP and we analyzed the DDR by ɣ-H2AX and 53bp1 staining in GFP positive cells (Fig.r13). Consistently, the results showed a decreased in the number of ɣ-H2AX and 53BP1 foci only when the wild-type version of LOXL2 was re-infected. Finally, in order to discard the possibility that LOXL2 could be affecting other DDR players to produce the same outcome, we forced chromatin condensation in the absence of LOXL2 expression.
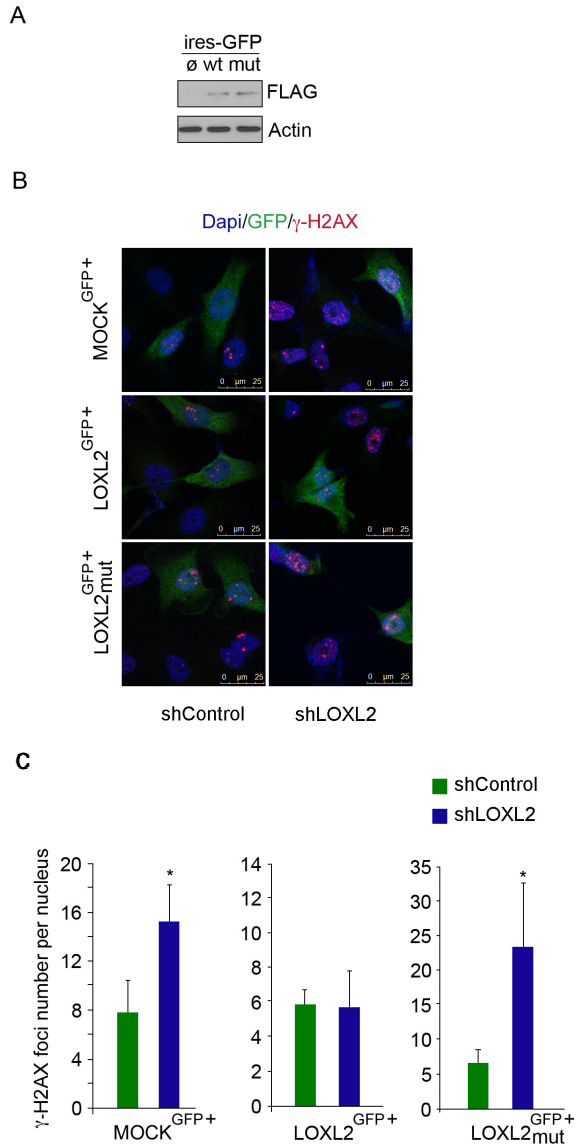
Figure.r13 LOXL2 reinfection recovers shControl DDR levels in shLOXL2 MDA-MB-231 cells due to its catalytically activity. (A) Western blot analysis from total extracts of 293 cells infected with the indicated retrovirus particle. Actin is used as a loading control. (B) ɣ-H2AX staining and foci quantification is showed by immunofluorescence with the indicated antibody. Graphs indicated changes in the number of the ɣ-H2AX foci in shControl and shLOXL2 MDA-MB-231 cells GFP positive cells. * p<0,05.

Since one of the main factors involved in chromatin condensation is the linker histone H1 (Woodcock, Skoultchi, and Fan 2006), H1-GFP was over expressed in shLOXL2 cells, and the DDR analyzed by counting ɣ-H2AX and 53BP1 foci. Interestingly, the number of foci in shLOXL2 cells reinfected with H1-GFP was restored as compared with the MOCK-GFP (Fig.r14A). These results suggest that LOXL2 activity oxidizing H3 leads to a chromatin condensation that impairs DDR activation. . Consistently, decrease in chromatin accessibility (closed chromatin) at selected genomic sites in LOXL2 knock-down cells upon H1-GFP over expression was confirmed by ATAC-qPCR of GFP-sorted cells (Fig.r14B). Importantly, clonogenic assays using GFP-sorted cells showed that recondensation of chromatin by H1 over expression in shLOXL2 cells partially blocked cell death (Fig.r14C).
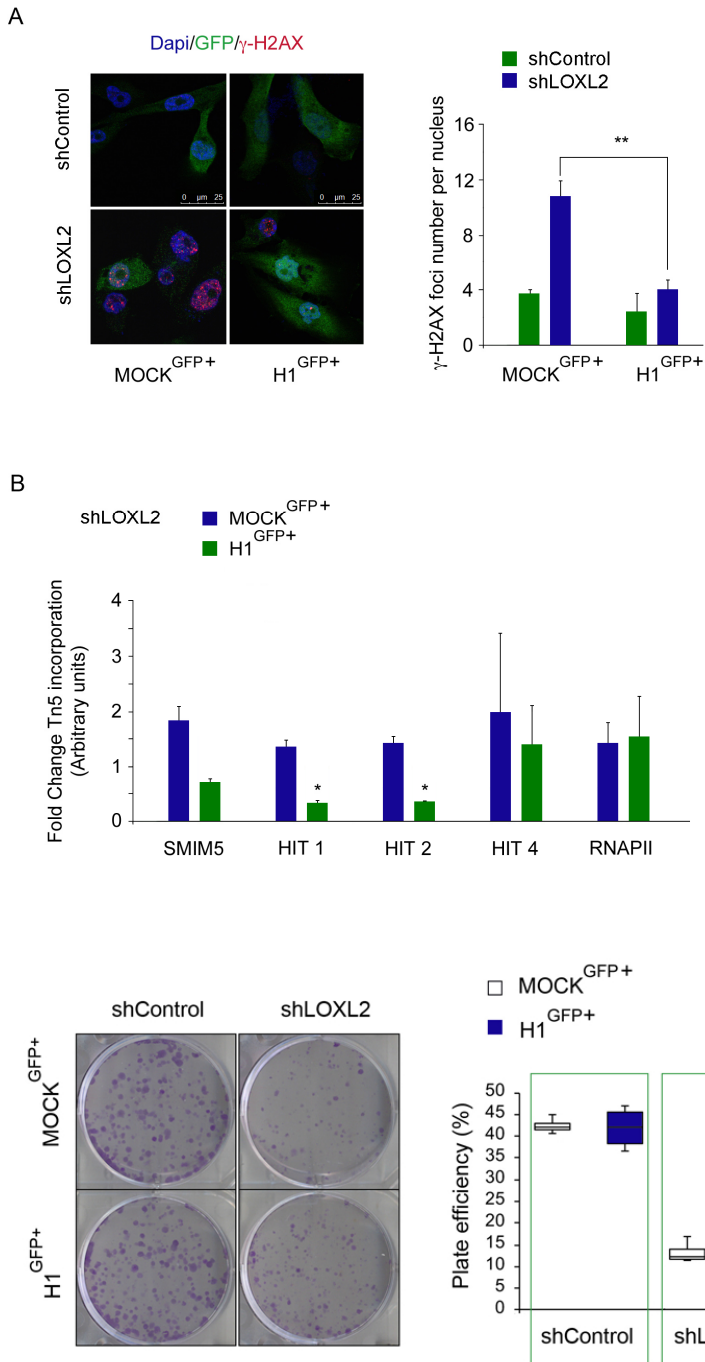
Figure.r14 Chromatin compaction induced by H1 infection recovers shControl DDR levels in shLOXL2 MDA-MB-231 cells. (A) γ-H2AX staining

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and foci quantification is showed by immunofluorescence. Graphs indicated changes in the number of the ɣ-H2AX foci in shControl and shLOXL2 MDA-MB-231 cells GFP positive cells. **p<0.01. (B) ATAC-qPCR was performed in sorted shLOXL2 GFP positive cells in order to assess chromatin compaction. qPCR amplification of the H3K4ox-selected hits were normalized to unchanging genomic region (HPRT) and expressed as the fold-change relative to the data obtained in shControl conditions, which were set as 1. RNAPII was used as a control. Error bars indicated standard deviation of at least three independent experiments. *p<0.05. (C) Colony-survival assay in GFP-sorted shControl and shLOXL2 MDA-MB-231 cells.

Moreover, in the RNA-seq analysis none of the 339 differentially expressed genes in shControl and shLOXL2 were associated with the activation of the DDR. Gene ontology (GO) analyses of those genes indicated that LOXL2 regulates genes involved in Wnt signaling pathway, cell adhesion, and morphogenesis. Furthermore, Gene Set Enrichment Analysis (GSEA) of specific signatures of the RNA-seq data showed an overrepresentation of genes involved in EMT (Fig.r15A-B).

A
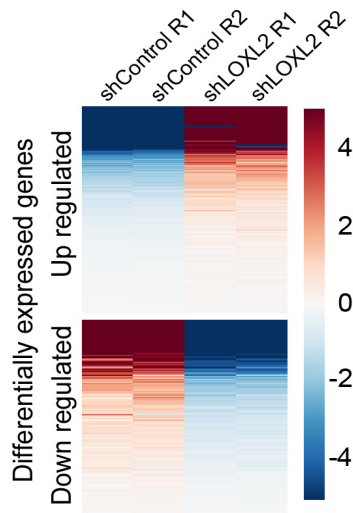
B

GO Biological Process

Name	P-value
regulation of canonical Wnt signaling pathway (GO:0060828)	0.0001139
digestive tract morphogenesis (GO:0048546)	0.0001694
regulation of cell adhesion (GO:0030155)	0.0002094
regulation of ossification (GO:0030278)	0.0003262
glial cell development (GO:0021782)	0.0003340
response to decreased oxygen levels (GO:0036293)	0.0003915
protein polymerization (GO:0051258)	0.0004199
regulation of Wnt signaling pathway (GO:0030111)	0.0004539
negative regulation of ossification (GO:0030279)	0.0004567
neural tube formation (GO:0001841)	0.0005241

Figure.r15 LOXL2 down-regulation in MDA-MB-231 cells did not alter DDR proteins by RNA-seq analysis. (A) Heat-map showing differentially expressed genes for each biological replicate in shControl and shLOXL2 (log2 fold change > 1, p < 0.01). (B) Gene ontology of differentially expressed genes in shLOXL2 vs shControl conditions.

4.7. TNBC MD-MB-231 cells are more sensitive to DNA lesions after LOXL2 down-regulation.

We then reasoned that inhibition of LOXL2 activity would induce a decrease in levels of H3K4ox, a more open chromatin conformation, contributing to expose the DNA lesions and therefore increasing cellular sensitivity, which could be a therapeutic opportunity to treat these highly resistant cells. In order to test this, we analyzed how shControl and shLOXL2 cells respond to ionizing radiation (IR) at low doses. γ-H2AX staining increased in shLOXL2 conditions at 1 hour after IR at 0,5 Gy (Fig.r16A). This increase in γ-H2AX staining could indicate either increased DSBs under these IR conditions or a higher level of signaling generated per DSB sites. Image analysis showed that the number of γ-H2AX foci was increased in shLOXL2 compared with shControl cells but the intensity per nucleus was not significant different than shControl cells. The increased number of dots and the absence of differences in intensity discarding an hyperactivation of the DDR signaling (Fig.r16A) suggest that in the absence of LOXL2 more DSBs are generated by IR at low doses. Furthermore, colony-survival showed that shControl cells are more resistant to irradiation than shLOXL2 (Fig.r16B). We then analyzed the DSB repair efficiency by quantifying the amount of γ-H2AX and 53BP1 foci loss by immunofluorescence after irradiation (Fig.r17A-B). The results showed a significant delay in DSB repair in shLOXL2 cells compared with shControl. Consistently, these results are in

agreement with the colony-survival assay showing that shControl cells are more resistant against cell death.
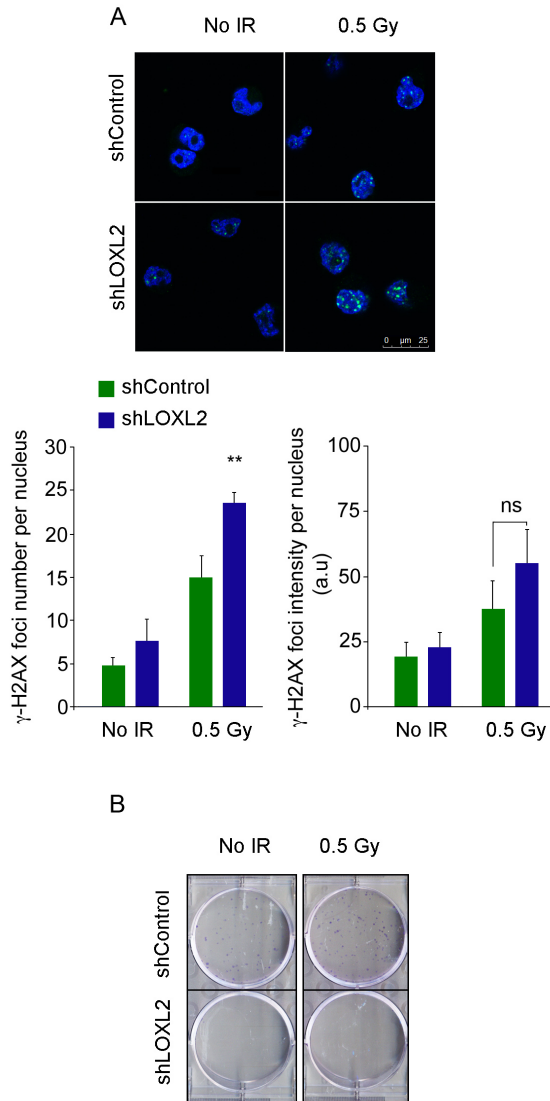
Figure.r16 Increased sensitivity in TNBC MDA-MB-231 cells after shLOXL2 down-regulation. (A) ɣ-H2AX staining and foci quantification and intensity are showed by immunofluorescence. Graphs indicated changes in the number of the ɣ-H2AX foci or intensity in shControl and shLOXL2 MDA-MB-

231 cells 1 hour after the indicated levels of irradiation. **p<0.01. (B) Colony-survival assay in shControl and shLOXL2 MDA-MB-231 cells under irradiation.
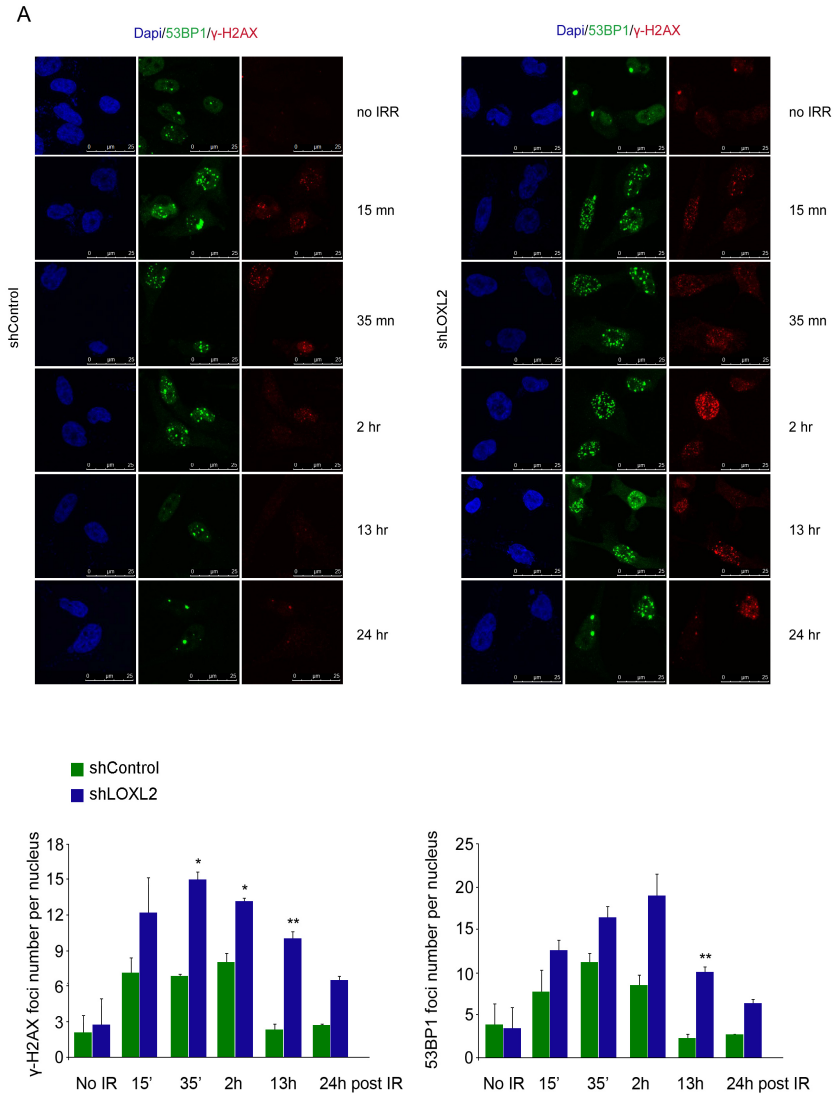
Figure.r17 TNBC MDA-MB-231 cells after shLOXL2 down-regulation show a delay in DSB repair. (A) ɣ-H2AX and 53BP1 staining and foci quantification is showed by immunofluorescence. (B) Graphs indicated changes in the number of the ɣ-H2AX and 53BP1 foci in shControl and shLOXL2 MDA-MB-231 cells at different points after 0.5 Gy irradiation. *<p0.05 **p<0.01.

4.8. H3K4ox is enriched in Triple Negative Breast Cancer Patient-Derived Xenographs (PDXs) compared to Luminal PDXs

Although experiments using cell lines have many limitations. For instance, a cell culture never has the heterogeneity of a solid tumor, the continuous cross-talk between the cells in a tissue and the surrounding environment is lost, the doubling potency has been altered, etc (Kaur and Dufour 2012). In addition, cell lines cultured in vitro did not contribute significantly to the discovery of targets that have had an important impact on patient survival (Williams et al. 2013). Since, we decided to check the levels of LOXL2 and H3K4ox in a panel of patient-derived xerography (PDXs) from different subtypes of breast cancer. By Western Blot analysis, LOXL2 and H3K4ox levels were higher in the TNBC PDXs (PDXs 8, 347, 385) compared with the luminal-origin ones (PDXs 71, 251M, 118), in agreement with our results in cancer cell lines (Fig.r18 A-B). In addition, ATAC-qPCR for selected H3K4ox regions showed less accessibility for the transposase in the TNBC PDX-8 compared to the luminal PDX-71 and did not in an irrelevant promoter (Fig.r18C).
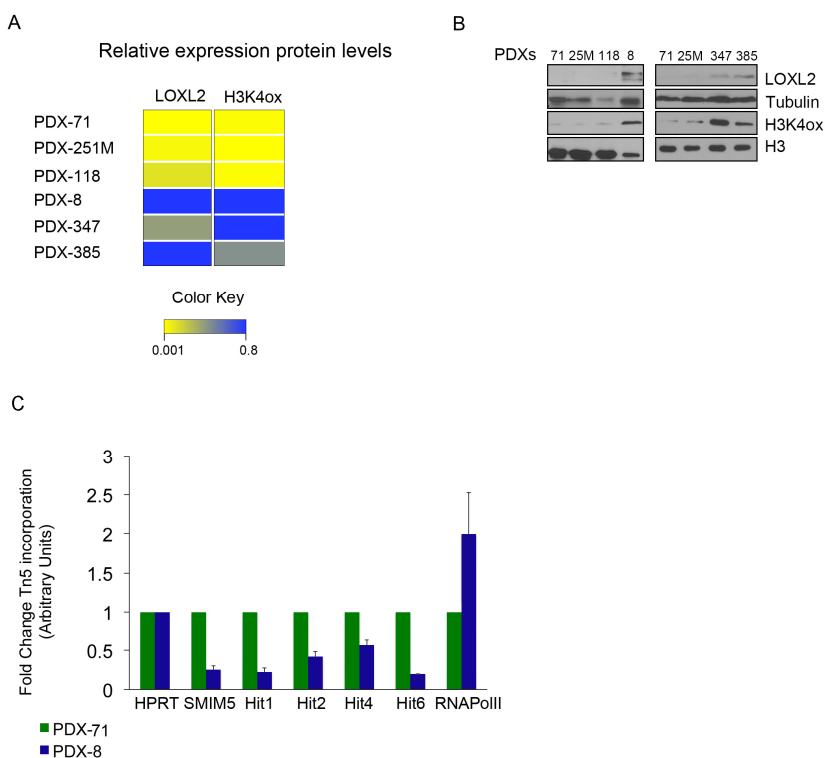
Figure.r18 Biological Significance of LOXL2 and H3K4ox Levels in a Panel of Breast Patient–Derived Xenographs (PDXs) (A) Heatmaps of LOXL2 and H3K4ox levels in a panel of breast PDXs (PDX-71 is as Luminal B breast tumor carrying a BRCA2 gene mutation; PDX-251M and PDX118 are Luminal B, HER2-positive breast tumors; and PDX-8, 347 and 385 are triple negative breast tumors). Westerns blots were digitally analyzed by integrating the density of each protein band and its corresponding loading control (see materials and methods). The ratio of the band intensity of LOXL2 or H3K4ox versus the band intensity of the corresponding loading control was defined as the relative protein expression level. The color key shown reveals the color-code used to visualize the relative protein expression levels, green correspond to low relative protein expression levels while red colors correspond to high relative protein expression levels. (B) Western blot analysis from the panel of breast PDX using the indicated antibody. Tubulin and H3 are used as a loading control. (C) ATAC-qPCR was used to assess open chromatin at selected genomic regions in PDX-71 and PDX-8. Data of qPCR amplification were normalized to unchanging genomic region (HPRT) and expressed as the fold-change relative to the data obtained in shControl conditions, which were set as 1.

5. DISCUSSION

In the following sections, I will discuss the genuineness of H3K4ox as an epigenetic mark (section 5.1), the relevance of its distribution in heterochromatic regions (section 5.2), the relation between LOXL2 and H3K4ox with the DDR (section 5.3), the importance of LOXL2 as a chromatin remodeler due to the generation of H3K4ox (section 5.4) and the value of targeting LOXL2 in oncologic therapies (section 5.5).

5.1. H3K4ox as a new epigenetic mark

In this work, we generate a new antibody that permitted us to identify the presence of oxidized lysine 4 in the tail domain of histone 3. The impossibility to generate an antibody against the final oxidized product of the LOXL2 due to the highly reactivity of the aldehyde group arises the necessity of an alternative approach. This was overcome using the TET enzyme reaction as an inspirational resolution (Fig.d1). TET enzymes remove the methyl groups from DNA by different independent oxidation reactions to first hydrolaze the 5-methylcytosine (5mC) and then the 5-hydroxilmethylcytosine (5hmC) to the 5-formylcytosine (5fC) (Münzel, Globisch, and Carell 2011; Kafer et al. 2016). Considering the similarities between the demethylation activity of LOXL2 and the activity of TET enzymes the use of the intermediate alcoholic form of the reaction arises as an interesting solution. Therefore, the use of the artificial aminoacid 6-hydroxinorleucine allows the

generation of a peptide that resembles to the intermediate alcohol product in the histone tail due to the LOXL2 activity.
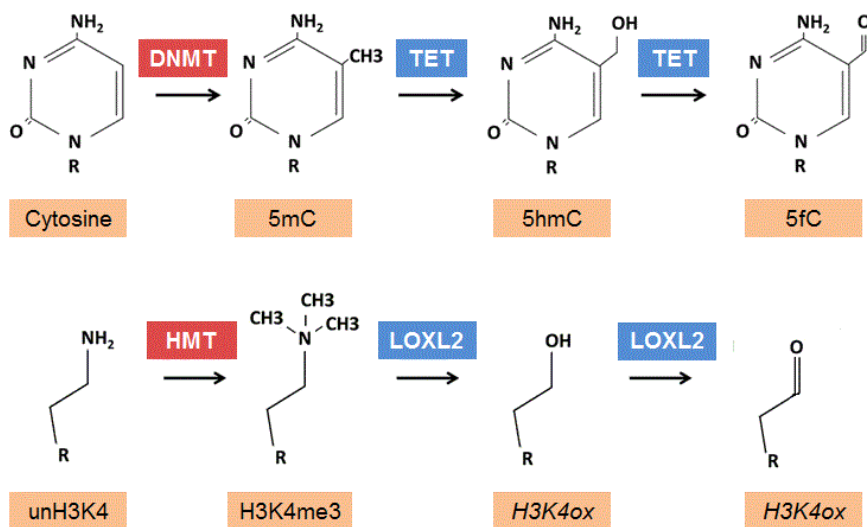
Figure.d1. Similarities between TET enzymes and LOXL2. Schematic cartoon highlighting the similarities between the reactions undergone by LOXL2 and TET enzymes. By the action of DNA methyltransefarses (DNMT), cytosine is methylated. The activity of the TET enzymes family eliminates this methylation by different oxidation reactions. Also lysines on the histone tail can be methylated by the activity of Histone Methyltransferases (HMT) enzymes. Here, it is LOXL2 who removes the trimethylated amino group promoting the intermediate alcoholic form that is rapidly oxidized by the LTQ cofactor of LOXL2 generating the aldehyde group. (Adapted from (Nightingale 2016)).

The use of the intermediate product of the reaction opens the door to different issues as for example the possibility of overestimate the distribution of the final product. However, according to the LOXL2 reaction, the LTQ domain rapidly converts the intermediate alcohol group to the final aldehyde group (Herranz et al. 2016). In addition, the results obtained using the antibody for the intermediate alcohol tightly correlate with the results prior obtain by our group determining the presence of the aldehyde group. Previously, in our

laboratory, a biotin-hydrazide approach has been used to determine the oxidation of histone 3. This approach relies on the ability of hydrazide to react with aldehyde groups and crosslink biotin. The in vitro experiments and also several ChIP experiments using the biotin-hydrazide approach tightly coincide with the results obtained with the generated antibody. So, the correlation between the two strategies allows us to back our antibody as a good tool to study the putative role of H3K4ox as an epigenetic mark.

In addition, as previously described, epigenetic marks in the histone tail are suitable to be recognized by other proteins. Temptingly, we assessed an in vitro experiment in order to elucidate the readers of the oxidized histone. Taking advantage of the recombinant LOXl2 proteins, H3K4me3 biotinylated peptide was oxidized using the mutant LOXL2 protein as a control. Then, peptides were recovered using streptavidin beads and incubated with nuclear extracts from 293T cells. Finally, the putative readers were obtained by mass-spectrometry (MS) analysis. Disappointingly, the results were not enough reliable and we discarded them. The use of recombinant protein does not actually guarantee a complete oxidation of the H3K4me3 peptide distorting, then, the results with readers of this mark. Thus, considering the use of the intermediate peptide and/or different nuclear extracts of breast cancer cells may be interesting steps to improve this approach. Indeed, the relevance of the readers of H3K4ox remains as an interesting future goal. The presence of different LOXL2 copartners with a chromatin-related activity (Herranz et al. 2016) also suggests that oxidized H3 can be considered as a new epigenetic mark. For example, CHAF1B,

HDAC1/2, RBBP4, EZH2, and Sin3A were described to be co-immunoprecipitated with LOXL2. Indeed, all of them are members of complexes implicated in the generation of a repressive environment in agreement with the repressive role of H3K4ox. As explained, different marks interplay to generate large stable environments, so H3K4ox could be a new epigenetic mark implicated in the constitution of large closed chromatin regions. Besides, previous work in our group described that oxidation of TAF10 by LOXL2, induces degradation of TAF10 (Iturbide et al. 2014). Nevertheless, how TAF10 oxidation induces its protein degradation is still unknown. Since oxidized TAF10 was prevented to interact with other members of the TFIID complex, the lack of partners could make it sensitive to degradation. Moreover, the presence of oxidized residues in different substrates does not need to have the same implications and although it leads to the degradation of TAF10 it could implicate also an epigenetic mark in histones. However, if oxidation of H3 induces histone degradation and incorporation of a new unmodified histone is still unknown. All things considered, oxidized H3 has to be considered as a suitable epigenetic mark.

5.2. H3K4ox heterochromatin distribution and relevance

Our results unveil the presence of H3K4ox in heterochromatic regions of MDA-MB-231 cells and how the presence of this oxidized histone favors the high compaction characteristic for

heterochromatin. Although the LOXL2 target H3K4me3 is described to be enriched in promoters (Santos-Rosa et al. 2002), LOXL2 activity in heterochromatin was previously described in our group. LOXL2 activity in pericentromeric regions of NMuMG cells under EMT process transiently down-regulates the transcription of long-noncoding major satellites (Millanes-Romero et al. 2013). In addition, this was crucial for the chromatin reorganization necessary for the transition from epithelial to mesenchymal state (Millanes-Romero et al. 2013; Casanova et al. 2013). Accordingly, LOXL2 activity emerges as a dual effect with different implications. Firstly, transcription and chromatin structure are altered by the deamination of H3K4me3 and secondly the release of an aldehyde group in the histone tail generates a specific chromatin environment.

Indeed, H3K4me3 is an important regulator of the cellular phenotype (Benayoun et al. 2014). Its deposition on the promoter of the different genes is related with the recruitment of the RNAPolII protein and consistent levels of transcription. Consequently, the regular transcription of different regions structures the chromatin from these transcription factories (Bortle and Corces 2017). It is possible that during tumor evolution, some cancer cells undergo EMT and start to express LOXL2. The transcription factor SNAI1, together with LOXL2, would participate in the permanent down-regulation of the CDH1 protein and in the transient heterochromatin transcripts inhibition, giving rise to the transformation of cancer epithelial cells into mesenchymal cells (Héctor Peinado et al. 2005; Schietke et al. 2010; Voloshenyuk et al. 2011). Accordingly, the

alteration of the established chromatin organization, such as by H3K4me3 deamination, arises as a necessary step for any cellular response that implies changes in cell phenotype and LOXL2 could be one reliable contributor to this alteration.

Moreover, the presence of H3K4ox alters the structure of heterochromatic regions. We demonstrated using ATAC-seq that the down-regulation of LOXL2 and the consequent loss of H3K4ox increase the accessibility of the Tn5 transposase to these H3K4ox positive regions with minor differences in the negative ones. Hence, our results suggest that the increase in LOXL2 levels generates an overrepresentation of H3K4ox in heterochromatin, thereby increasing its compaction. Still, the role of H3K4ox promoting chromatin compaction is unclear and several explanations could be going on. Certainly, the loss of H3K4me3 causes the displacement of different chromatin factors that contribute to an increased chromatin accessibility, such as CHD1 (chromo-ATPase/helicase-DNA-binding 1) (Flanagan et al. 2005) or NURF (Nucleosome Remodelling Factor) (H. Li et al. 2006). Even, LOXL2 reaction could disturb the electric charge of the histone tail since the loss of the amino group by the deamination diminishes positives charges (Hatasa et al. 2016). However, these changes do not reveal as relevant as they may promote a chromatin opening due to the loss of interaction between a positive histone tail and a negative charged DNA as happens with the addition of acetyl groups by histone acetyl transferases (HAT) (Bannister and Kouzarides 2011). Although, H3K4ox could promote lysine cross-link between histones as it happens in the collagen fiber generation. Indeed,

aldehyde groups are very reactive and easily interact with different chemical groups as other aldehydes (Yamauchi and Sricholpech 2012) or amino groups (Takahashi 1977). Moreover, as a putative epigenetic mark, H3K4ox could be recognized by different proteins related with chromatin compaction. In fact, current work in the laboratory unveils the relation between H3K4ox and the ATP-dependent DNA helicases RUVBL1 and RUVBL2 that may promote the deposition of the histone variant H2A.Z with an important role in chromatin structure (Rege et al. 2015) opening an interesting line of investigation. Indeed, a possible role by which H3K4ox would promote histone deposition fits excellently with the relevance of the deposition of histone variants like H2A.Z or H3.3 in heterochromatin. Certainly, H2A.Z and H3.3 are enriched in heterochromatin (de Castro et al. 2017) and their deposition contribute to the pericentromeric heterochromatin organization during early development (Corpet et al. 2014; Rangasamy et al. 2003; Santenard et al. 2010). Finally, as explained, LOXL2 copartners included several chromatin remodelers implicated in repression as histone deacetylases (Herranz et al. 2016). Hence, LOXL2 copartners could contribute to the less accessibility since deacetylation in the histone tail increases the chromatin compaction (Eskeland et al. 2010). Absolutely, further experiments are necessary to elucidate the different contribution of these different hypotheses.

As described, chromatin accessibility approaches allow inferring direct effects of chromatin structure modifications on cellular biology. Together with ChIP-seq data, they become a powerful tool

to link regulatory elements with disease phenotypes and the assessment of clinical samples for epigenetic biomarkers of disease (Tsompana and Buck 2014b). Increasing number of publications use the ATAC-seq approach in order to infer the importance of chromatin structure and occupancy (Buenrostro, Wu, Litzenburger, et al. 2015; Giorgetti et al. 2016; J. Wu et al. 2016). A recently published surprising variation of the technique used the transposase activity to selectively and covalently insert fluorophores (ATAC-see) at open chromatin sites genome wide allowing visualization by common immunofluorescence (IF) (Chen et al. 2016). In the future, these approaches will allow the identification of the unique spatial organization of the accessible genome and the changes of the chromatin structure between the different human cell types in normal and pathogenic situations. Interestingly, studying the role of LOXL2 using the ATAC-see approach could be an interesting tool to visualize by immunofluorescence the effects of LOXL2 in the TNBC cells. For example, the down-regulation of LOXL2 may enhance the ATAC-see signaling and the immunofluorescence could enlighten the distribution of the newly opened regions. Also, IF of the fluorophores of the ATAC-see in the opened regions with the DNA damaged signals such as 53BP1 or γ-H2AX will allow a better understanding of the chromatin structure relevance in the DNA damage response signaling.

Recently, different groups have suggested the importance of maintaining the compaction of heterochromatin in order to prevent an increase of the expression of noncoding RNAs produced from heterochromatin sequences, the genomic instability associated with

heterochromatin dysfunction (Grézy et al. 2016; Molina et al. 2016; Postepska-Igielska et al. 2013) and to prevent alterations in the cellular fate (Boonsanay et al. 2016; Gonzalez-Sandoval et al. 2015). Indeed, the presence of H3K4ox is related with major satellite repression during the EMT (Millanes-Romero et al. 2013). Clearly, upon TGF-β induction of EMT, SNAI1 is rapidly upregulated, binds to pericentromeric regions, and recruits LOXL2 to oxidize H3 and repress major satellite transcription enabling chromatin reorganization and acquisition of mesenchymal traits. Moreover, two different groups have suggested that EMT is dispensable for lung and pancreas initial metastasis but contributes significantly to the formation of recurrent metastasis after chemotherapy (Fischer et al. 2015; X. Zheng et al. 2015). Considering, these findings agree with our results, in which we observed how resistance shown by MDA-MB-231 breast cancer cells is linked to both the mesenchymal phenotype and the increase in H3K4ox levels in heterochromatin.

5.3. LOXL2 and H3K4ox prevent activation of DDR

Strikingly, our results disclose the relation between activation of the DDR with LOXL2 expression, H3K4ox levels and chromatin compaction. Certainly, chromatin compaction would allow the accumulation of mutations and DNA lesions in these cells, which could be linked with increased tumor aggressiveness (Schuster-Böckler and Lehner 2012; Supek and Lehner 2015). In TNBC cells,

this chromatin compaction seems to be induced by high H3K4ox levels and increased LOXL2 expression. Notably, the LOXL2 activity also leads to an increase in H_2O_2 production contributing to a more stressing environment. In fact, H_2O_2, like other reactive oxygen species (ROS), is a largely described DNA damage agent (Jena 2012; Cadet and Wagner 2013) moreover this is not relevant in our model because increased damage is observe in the absence of LOXL2 where less H_2O_2 is produced. Hence, the production of a ROS agent could be considered as a lesser evil in the TNBC with high LOXL2 protein levels. Nevertheless, the activation of the DDR machinery when chromatin acquires a more open state in the absence of LOXL2 could be due to DNA lesions that are either already present in these cells or newly formed by the cellular stress generated by the new chromatin conformation. Still, current work in the laboratory using the comet assay experiment (data not shown) do not show differences between control and LOXL2 knock-down cells suggesting that the absence of LOXL2 does not generate new damage. Indeed, our results in rescue experiments highlight the relevance of the chromatin structure and the importance of H3K4ox in this conformation. The rescue experiment using the wild-type and the catalytic mutant of LOXL2 clearly showed how the presence of the catalytically active form of LOXL2 impairs the DDR signaling. Although as LOXL2 has several substrates besides histones related with DNA damage (Iturbide et al. 2014) we cannot discard a role for LOXL2 in the direct regulation of the DDR machinery. For example, LOXL2 is suggested to oxidize MRE11 and RAD50, two proteins of the MRN complex. As previously explained, this

complex has a crucial role in identifying the DSB presence in the genome. As described for TAF10 protein, the oxidation of these proteins could be altering its interaction and the complex formation impairing its main function detecting DSB. Accordingly, it cannot be discard that the down-regulation of LOXL2 could alter the activity of a putative reader of H3K4ox implicated in DDR. Furthermore, the absence of H3K4ox mark could alter the recruitment of a putative reader related with the impairment of DDR. For example, the recruitment of the nuclear receptor NR1D1a to the DSB sites impairs the recruitment of DDR proteins (Ka et al. 2017). However, the fact that the DDR can be inhibited by only forcing the compaction of chromatin—H1 overexpression in the absence of LOXL2—suggests that the main molecular mechanism of LOXL2 diminishing the DDR signaling is to induce chromatin compaction.

The correlation between chromatin compaction and DDR has been largely reported in the literature and different theories highlight the importance of establishing a closed or an open chromatin environment after a DNA lesion (Cann and Dellaire 2011; M. L. Li, Yuan, and Greenberg 2014). Thus, chromatin compaction during DNA damage repair is likely to be dynamic both at the break and in the surrounding chromatin neighborhood. In addition, different strategies are proposed in order to repair euchromatin or heterochromatin (Goodarzi et al. 2008; Gursoy-Yuzugullu, House, and Price 2016). Indeed, the relation between chromatin compaction and DDR is controversial. For example, persistent chromatin compaction was recently described to promote DDR signaling

(Burgess et al. 2014). Interestingly, while the persistent chromatin compaction enhanced DDR activation after irradiation-induced breaks, it reduced the recovery and survival of those cells. Still, an open chromatin state in glioma cancer cells enhances DDR signal (Murga et al. 2007). This chromatin decompaction generated by H1 down-regulation or using histone deacetylase inhibitors was described to enhance DDR signal. In agreement, H1-depleted cells are hyperresistant to DNA damage lesions and show hypersensitive checkpoints. These characteristics are explained by a transient increase in the amount of signaling generated by an external DNA break (Bao et al. 2006). Accordingly, impairing DDR signaling by compacting chromatin (Murga et al. 2007) or by blocking checkpoint kinase activity could be a source of radiosensitization in glioma cancer cells (Bao et al. 2006). However, according to our results this is not the case for TNBC cells; rather, for these cells, chromatin compaction seems to be one of the players directly responsible for acquisition of resistance. Actually, the importance of chromatin compaction in preventing the DNA lesions was highlighted by studying the different distribution of DSB produced by ionizing radiation in a comparative study with human embryonic stem cells (hESC) and a set of differentiated cell lines (Venkatesh et al. 2016). Remarkably, hESC cells show considerably more DSB than differentiated derivate cell lines and this correlates with the increased H3K9me3 staining in the differentiated ones (Venkatesh et al. 2016) according to the loss of chromatin accessibility during differentiation (Meshorer et al. 2006). Strikingly, hESC cells differentiation into neuronal progenitor stem cells by retinoic acid

(RA) induction requires LOXL2 activity and increases H3K4ox levels (Iturbide et al. 2014) suggesting the relevant role of H3K4ox in chromatin compaction and DDR skipping.

Finally, the promotion of translocations by DSB highlights the link between DDR and chromatin structure (Ghezraoui et al. 2014). Translocations have been largely associated with cancer and learning how to face them may open new therapeutic opportunities (Hromas et al. 2016; J. Zheng 2013). Interestingly, these translocations are able even to draw an interaction map of the genome of a cell just by their frequency (Yu Zhang et al. 2012). Hence, translocations are generated by DSB and this is confirmed by direct-targeted nuclease DSB (Weinstock, Brunet, and Jasin 2008; Frock et al. 2014). Also, these translocations are promoted by the recruitment of nuclear myosin 1 (NM1) by γ-H2A.X, leading to chromosome territories relocation during DNA repair (Kulashreshtha et al. 2016). Interestingly, it has been also reported that increased levels of transcription moderate the frequency of mutations in H3K9me3-dense regions by reducing the limitations imposed by chromatin structure on the DDR (C. L. Zheng et al. 2014). To sum up, these works demonstrate the importance of chromatin compaction for DNA protection and DNA damage repair (Qiu 2015) and here we demonstrated that, in addition to H1 levels, oxidation of H3 by LOXL2 is another molecular mechanism that induces compaction in MDA-MB-231 cells and that this compaction exhorts a protective role.

5.4. LOXL2 as a chromatin remodeler

The relevance of LOXL2 in cancer and the acquisition of cellular malignancy has been largely described, since it is overexpressed in many tumors (Fong et al. 2007; Moreno-Bueno et al. 2011; Héctor Peinado et al. 2008; Torres et al. 2015; Wong et al. 2014) and has an important role in tumor formation (Martin et al. 2015). A putative role of LOXL2 as a chromatin remodeler involved in cellular differentiation arises from our results. Considering the cancer stem cell (CSC) paradigm (Singh et al. 2015), based on the observation that distinct tumor cell populations are uniquely capable of tumor growth in serial passages, LOXL2 arises as a putative chromatin remodeler involved in cellular survival. In addition, as previously explain, LOXL2 fully activation after RA-induction is a necessary step in embryonic stem cells differentiation into neuronal progenitor cells, diminishing the expression of the different embryonic genes and increasing H3K4ox levels (Iturbide et al. 2014). Indeed, it could be also related to the acquisition of a more closed chromatin conformation. Actually, LOXL2 could also play a role during the differentiation of CSCs towards progenitor cancer-initiating cells, with an increase in the levels of oxidized H3 conferring an evolutionary advantage by inhibiting the DDR in heterochromatin. Several examples of a putative differentiation role for LOXL2 have been described. In addition to the already mentioned differentiation of embryonic stem cells, LOXL2 overexpression in skin carcinogenesis depends on its ability to negatively modulate epidermal differentiation by repressing the

NOTCH1 promoter activity (Martin et al. 2015). Also, overexpression of nuclear LOXL2 in MCF-7 cells, a luminal A noninvasive breast cancer cell, promotes a rapidly transition to a mesenchymal phenotype (Moon et al. 2013; Hollosi et al. 2009). Together, these observations suggest that chromatin compaction induced by LOXL2 in order to bypass the accumulation of DNA lesions is a part of the differentiation program. Besides, the importance of chromatin compaction in differentiation is increasingly relevant (B Wen, Wu, and Shinkai 2009; McDonald et al. 2011; Ricci et al. 2015). According to the RNA-seq data shown here, the relevance of LOXL2 and the compaction of the chromatin by the presence of H3K4ox induce the expression of a set of EMT-related genes. Remarkably, chromatin accessibility studies in a set of cells with specific differentiation rates (Gomez et al. 2016; Buenrostro, Wu, Litzenburger, et al. 2015; Cusanovich et al. 2015) and the loss of hyperdynamic plasticity of chromatin proteins after differentiation of pluripotent ESC (Meshorer et al. 2006) are some examples that highlight the role of chromatin compaction in cellular differentiation.

Moreover, the hypothesis of a specific chromatin compaction profile for each cell opens the possibility for a highly-compacted chromatin-containing nucleus as a characteristic of the/a mesenchymal phenotype. In fact, the relevance of mechanical perturbations on chromatin compaction is clear in mesenchymal stem cell differentiation (Heo, Driscoll, et al. 2016). The establishment of a baseline level of cellular contractility is necessary for chromatin condensation and TGF-β signaling in

response to whole-cell mechanical alterations. Hence, specific chromatin condensation patterns may favor the expression of specialized sets of genes, rendering particular cell types finely-tuned to respond to the mechanical stimuli they experience (Heo, Han, et al. 2016). Remarkably, chromatin compaction has been described as a promoter of cellular migration. In breast cancer cells, the highly metastatic line MDA-MB-231 showed higher transmigration capabilities than the poorly metastatic MCF7. Since chromatin decondensing drug MTA reduced the transmigration efficiency of MDA-MB-231 cells along microchannels in a width-dependent manner, the mesenchymal phenotype may be related to the chromatin condensation status (Yi Fu et al. 2012). Also, altering the levels of H4K20me3 and H3K9me3 heterochromatin marks correlated with migration capacity. Interestingly, breast cancer cells ectopically overexpressing the methyltransferase SUV420H1 or SUV420H2, increasing H4K20me3, showed suppressed cell invasiveness, whereas knock-down of SUV420H2 in vitro activated a normal mammary epithelial-cell invasion (Yokoyama et al. 2014). Besides, overexpressing SUV39H1, the methyltransferase writer of H3K9me3, in the same cell lines activated cell migration and these effects are absolutely impaired using SUV39H1 mutant (Yokoyama et al. 2013). Finally, in a different EMT-associated context, wound healing, cells in the edge of the wound showed increased heterochromatin markers like H3K27me3 and H4K20me1 compared to cells far from the wound edge. In addition, those cells closer to the edge of the wound showed increased resistance to DNaseI activity in comparison to cells far from the wound edge

(Gerlitz and Bustin 2010). Accordingly, cells closer to the wound edge show more compacted chromatin than the distant ones.

5.5. Targeting LOXL2 as a therapeutic target

Importantly, we have shown that patient-derived samples from TNBC tumors have the same behavior than MDA-MB-231 breast cancer cells; specifically, as observed for the cell line, TNBC tumors express more LOXL2, have higher H3K4ox levels, and show more compacted chromatin compared to luminal tumors in the ChIP-H3K4ox positive regions by ATAC-qPCR. Indeed, assessing the global chromatin structure using genome-wide ATAC-seq data will be a necessary step to further characterizes these tumors. This makes LOXL2 a good candidate as a therapeutic target in these TNBC tumors. Certainly, on one hand, the development of inhibitors of LOXL2 could be a promising success. Moreover, several inhibitors have been described for LOXL2 (Chang et al. 2017; Hutchinson et al. 2017). However, the effects of these inhibitors alter the extracellular activity of LOXL2 and do not focus on the importance of LOXL2 and H3K4ox neither for compaction nor for DDR signaling. Accordingly, our group is working on the characterization of a putative LOXL2 inhibitor, zonisamide. Interestingly, zonisamide is a described anti-epileptic generic drug that is already used in humans and has been suggested as a putative LOXL2 inhibitor by a drug-repurposing approach. Hence, the zonisamide inhibits in vitro the enzymatic activity of human recombinant LOXL2 enzyme, and that such inhibition decreases the

proliferative capacity of breast cancer cells in vivo and causes cell death. Breast cancer triple negative cells with high LOXL2 levels died after 4 days of zonisamide treatment. On the other hand, a relevant humanized monoclonal antibody against LOXL2, simtuzumab, has been tested for the treatment of aberrant fibrosis (Meissner et al. 2016). Disappointingly, it has been stopped the phase 2 trials for idiopathic pulmonary fibrosis (IPF) due to the lack of results (Raghu et al. 2017).

Considering, our results suggest that using inhibitors that could affect chromatin conformation as adjuvants in conventional therapy would provide novel therapeutic opportunities for TNBC tumors. Indeed, recent different studies support the combination of inhibitors of chromatin compaction with conventional therapy. First, the use of a hypomethylation therapy (HMT) impairs MDA-MB-231 cells altering the levels of H4K20me2 and H3K79me2 and sensitizes them to IR or to the DNA-damage inducer cisplatin, leading to apoptosis (Montenegro et al. 2016). Also, the use of histone deacetylases inhibitors (HDACi) combined with PARP inhibitors impair the DDR, leading cells to apoptosis (Ha et al. 2014).

Summarizing, impairing H3K4ox accumulation with LOXL2 inhibitors alters chromatin compaction. This enhances DDR signaling and sensitizes TNBC to conventional therapies. Therefore, using LOXL2 inhibitors as adjuvants to present therapies is a promising strategy for treating TNBC tumors.

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