Mammalian Trx2 function

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Zusammenfassung

Mitglieder der trxG und PcG Proteinfamilie in *Drosophila* sind epigenetische Regulatoren, deren Aktivitaet fuer die Aufrechterhaltung eines ordnungsgerechten Genexpressionsmusters der homeotischen Gene waehrend der Embryonalentwicklung der Fliege verantwortlich ist. Beide Proteinfamilien erreichen ihre entgegengesetzten Wirkungsweisen durch Einflussnahme auf den aktivierten oder reprimierten Chromatinstatus der Zelle. Waehrend fuer MLL als Saeugerprotein aus der trxG Familie schon nachgewiesen wurde, dass es die Expression der Hoxgene beeinflusst, ist Wirkungsweise, Zielgruppe und zellulaere Lokalisation des zweiten bekannten Saeugetierhomologs TRX2 noch weitestgehend unerforscht. Der Schwerpunkt dieser Arbeit stuetzt sich deshalb auf die Untersuchung der Grundfunktionen des Saeugerproteins TRX2 waehrend der Embryonalentwicklung der Maus. Das Gelingen der Herstellung einer embryonalen Stammzellinie (ES) die einen homozygoten knock-out des Trx2 Gens traegt, und deren Analyse ergab, dass Lebensfunktionen und Zellzykluskontrolle im totipotenten Stadium der ES Zelle durch den Verlust des TRX2 Proteins nicht beeintraechtigt sind. Eine N-terminale Fusionierung von TRX2 an das gelb-fluoreszierenden Proteins (EYFP) diente ausserdem als Werkzeug, um die subzellulaere Lokalisation des Proteins in ES Zellen zu studieren. Die Analyse des hypomorphen Phenotyps von EYFP-Trx2 homozygoten Maeusen ergab, dass intaktes TRX2 auch waehrend spaeter Phasen der embryonalen, foetalen und adulten Entwicklung noetig ist. Diese Beobachtung wurde weiter durch Injektionsexperimente von Trx2-/- ES Zellen in Blastocysten unterstuetzt. Schwach chimaere Embryonen, die aus dieser injizierten Blastozyste hervorgehen, zeigen eine variable aber allumfassende Mitwirkung der knock-out Zellen bis zum embryonalen Stadium E10.5. Allerdings zeigte sich dann eine zunehmende Eliminierung der Trx2-/- Zellen bis zum Embryonalstadium E18.5. Sobald die Beteiligung der -/- Zellen in den untersuchten Embryonen einen bestimmten Grenzwert ueberschritt, zeigten solch hoch-chimaeren Embryonen einen Phenotyp, der den von Trx2 knock-out Embryonen widerspiegelt. Durch diese Beobachtung kann gefolgert werden, dass die fehlerhafte Embryonalentwicklung nicht durch einen Defekt im extraembryonalen Gewebe, sondern durch das Fehlen von TRX2 im eigentlichen Embryo hervorgerufen wird.

Alle Experimente deuten auf einen kontinuierlichen, zelltypunspezifischen und zellautonomen Bedarf an TRX2 waehrend der Embryonalentwicklung von Maeusen hin.

E. Einleitung

Die fehlerfreie Expression homeotischer Gene ist für die Festlegung von Zellidentitäten entlang der Körperachse eines sich entwickelnden Embryos unbedingt erforderlich. Eine gestörte Regulation homeotischer Gene führt zur Transformation eines Körpersegments in ein Anderes. Die maternalen Produkte von Segmentations-, Pair-rule- und Gap-Genen etablieren spezifische Muster homeotischer Genexpression, welche während der Entwicklung durch zwei gegensätzliche Regulatorgruppen, der Trithoraxgruppe (TrxG) und der Polycombgruppe (PcG), aufrecht erhalten werden. PcG-Proteine gelten als reprimierende Faktoren, während TrxG Proteine verantwortlich für die Aufrechterhaltung aktiv expremierter Abschnitte sind. Trx- und Pc-Proteingruppen erwirken den beobachteten Effekt auf ihre Zielgene vermutlich durch die Einrichtung von transkriptional permissiven bzw. inhibitorischen Chromatindomänen (Mamoudi und Verrijzer, 2001; Paro et al., 1998; Pirotta, 1998; Gould, 1997).

Die vorliegende Arbeit befaßt sich hauptsächlich damit, die Funktionsweise des Säugetierproteins TRX2 zu untersuchen. Da grundlegenden Erkenntnisse zur Wirkweise von TrxG- und PcG-Proteinen aus Forschung an der Fruchtfliege *Drosophila melanogaster* stammen, soll zu Beginn dieses Kapitels eine Darstellung der bisher bekannten Eigenschaften des *Drosophila* Proteins TRX stehen.

DIE TRX-G IN *DROSOPHILA MELANOGASTER*

E-1   Trx

Das Trithorax Gen (trx) in *D. melanogaster* codiert für ein großes Protein (TRX), dessen kontinuierliche Expression erforderlich für eine normale Embryonalentwicklung der Fliege ist (Ingham, 1981; 1985). Trx wurde 1968 von Lewis als Regulator des Bithorax Proteins entdeckt. Inzwischen konnte gezeigt werden, daß trx für die korrekte Transkription der homeotischen Gene innerhalb des Antennapedia- und des Bithorax-Genkomplexes (ANT-C und BX-C) verantwortlich ist. Trx Phänotypen beinhalten

homeotische Transformationen, in denen Körpersegmente die Identität meist weiter anterior gelegener Segmente annehmen. Die meisten Mutationen innerhalb des trx Gens führen schon im Zygotenstadium zur Lethalität. Einige weniger schwerwiegende Mutationen erlauben zwar eine Entwicklung zum adulten Tier, allerdings zeigen die daraus hervorgehenden Mutanten die oben angesprochenen homeotischen Transformationen. So entsteht beispielsweise am dritte Thoraxsegment anstatt von Halteren ein Flügelpaar, das normalerweise im zweiten Thoraxsegment angelegt wird. In einem anderen Fall transformiert das Segment, welches bei männlichen Fliegen üblicherweise die Geschlechtsorgane trägt in das anterior gelegene Segment und entwickelt ein Beinpaar. Namensgebend war allerdings eine für die bekannten Trx Allele unübliche bidirektionale Transformation, die durch ein hypomorphes Allel hervorgerufen wird. In diesem Fall transformiert sowohl das erste als auch das dritte Thoraxsegment und beide nehmen die Identität des zweiten Thoraxsegments an, so daß Fliegen mit dreifachem Thorax (**tri**thorax) geboren wurden (Ingham, 1998). Diese bidirektionale Transformation ist keinesfalls als eine gemeinsame Eigenschaft aller Trx Allele anzusehen, dennoch bleibt sie als namensgebende Beschreibung erhalten.

Die zytogenetische Region von trx erstreckt sich über 40 kb auf dem rechten Arm von Chromosom 3 (Region 88B) (Mozer and Dawid, 1989; Breen und Harte, 1991). Die 25 kb große Transkriptionseinheit besteht aus 8 Exons, und codiert für zwei isoforme TRX Proteine: TRXI (3358 AS) und TRXII (3726 AS). Die beiden Isoformen unterscheiden sich in 368 N-terminalen Aminosäuren, die von den alternativ genutzten Exons 2 und 3 codiert werden (Mazo et al., 1990; Breen und Harte, 1991; Sedkov et al., 1994; Stassen et al., 1995). Mindestens fünf alternativ gespleißte TRX Formen existieren:

- Die 10 kb große TRXI codierende mRNA wird den Eizellen maternal beigefügt und ist lediglich in 0-3 Stunden alten Embryos nachweisbar (Mozer und Dawid, 1989; Breen und Harte, 1991; Sedkov et al., 1994).
- Eine ebenfalls TRXI codierende mRNA, die hauptsächlich in Embryonen des Blastodermstadiums vorkommt und deren Vorkommen im weiteren Entwicklungsverlauf stetig abnimmt, ist 12 kb groß. (Mozer und Dawid, 1989; Breen und Harte, 1991; Sedkov et al., 1994).

- TRXII wird von einer 14 kb großen mRNA codiert, die von der embryonalen Phase der Keimbandverlängerung bis hin zum Puppenstadium vorkommt, und die einzige während imaginaler Zellproliferation transkribierte RNA Form ist (Mozer und Dawid, 1989; Breen und Harte, 1991; Sedkov et al., 1994).

Die zygotischen RNAs werden in frühen Entwicklungsstadien ubiquitär transkribiert, in späteren Stadien der Embryonalentwicklung ist die trx RNA hauptsächlich im ZNS nachweisbar (Mozer et al., 1989). Western-Blot-Analysen haben gezeigt, daß TRXI in frühen Stadien die überwiegende Isoform darstellt, während die TRXII-Isoform im letzten Drittel der Embryogenese vorherrschend ist. (Kuzin et al., 1994).

E-2   TRX Zielgene und Response-Elemente

Übereinstimmend mit den phänotypischen Beobachtungen hervorgerufen durch trx Mutationen, gehören homeotische Gene wie zum Beispiel *Ultrabithorax, Abdominal-A, Abdominal-B, Antennapedia, Sex combs reduced* und *Deformed* zu den Ziel-Genen von TRX (Mazo et al., 1990; Breen und Harte, 1993; Sedkov et al., 1994). Neben den homeotischen Gene sind auch die Gene der Transkriptionsfaktoren *engrailed* (Breen et al., 1995) und *fork head* (Kuzin et al., 1994) von der Wirkweise des TRX Proteins abhängig.

Weitgehend unbekannt sind nach wie vor die Mechanismen, welche die Bindung der PcG und TrxG Proteine an ihre Zielgene ermöglichen. Obwohl für die meisten charakterisierten PcG und TrxG Proteine keine sequenzspezifische DNA-Bindefähigkeit bekannt ist, konnten durch genetische und biochemische Untersuchungen DNA-Elemente identifiziert werden, welche auf PcG und TrxG Proteine reagieren (**T**rxG und **P**cG **R**esponse **E**lements; TREs und PREs). In den regulierenden Regionen der Gene *ultrabithorax* und *sex combs reduced* wurden TREs und PREs gefunden, die allerdings keine eindeutige Struktur haben, und nur wenige signifikante DNA-Sequenz-Motive aufweisen (Gair und Garcia-Bellido, 1990; Chan et al., 1994; Chang et al., 1995; Gindhart und Kaufmann, 1995). TREs und PREs liegen auf der DNA sehr eng beieinander (Tillib et al., 1999), aber ihre Wirkweisen können klar voneinander abgegrenzten DNA-Elementen zugeordnet werden. Diese räumliche Nähe spricht für

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eine direkte Interaktion der regulatorischen Einheiten, aber gegen eine unmittelbare Konkurrenz der gegensätzlich wirkenden Faktoren um Zielsequenzen. Übereinstimmend hiermit stellte sich heraus, daß TRX an spezifischen Stellen polytäner Riesenchromosomen bindet, und an vielen dieser Stellen mit PC colokalisiert (Chinwalla et al., 1995).

E-3   Die Proteine und Proteinkomplexe der trx-G

Die Klassifizierung, nach der Genen eine Mitgliedschaft in der trxG zugesprochen wird, ist variabel. Verschiedene genetische Kriterien wurden bisher verwandt:

- Zeigt ein Gen-knock-out einen homeotischen Phänotyp, der einer "loss-of function" Mutation homeotischer Gene gleicht,
- Verstärkt die Mutation eines Genes den Phänotyp einer anderen trxG-Mutante,
- Wirkt die Mutation eines Genes dem Phänotyp einer PcG-Mutante entgegen,

so wird dieses Gen der trx-Gengruppe zugeordnet.

Die trxG ist eine heterogene Gruppe deren Mitglieder sind an den unterschiedlichen Regulationsschritten zur Aufrechterhaltung der homeotischen Genexpression beteiligen. Da sich die Wirkungsweisen von trxG Proteinen signifikant unterscheiden, kann man ein Modell, in dem alle trxG Genprodukte in einem einzigen Mechanismenkomplex zusammenarbeiten, ausschließen. TrxG Proteine lassen sich in wenigstens drei funktionale Untergruppen aufteilen:

1. TrxG I: Brahma und die Verbindung zum Swi/Snf Komplex
2. TrxG II: GAGA-Faktor und Zeste
3. TrxG III: Trx, ash1 und ash2 als Histon-modifizierende Faktoren

E-3.1 TrxG I: Brahma und die Verbindung zum Swi/Snf Komplex

Der Brahma-Proteinkomplex enthält die trxG Proteine Brahma (BRM) und Moira (MOR), das Snf5 verwandte Protein SNR1 (**S**nf5 **R**elated **P**rotein) und vier weitere Brahma-assozierte Proteine (**B**rahma **A**ssociated **P**roteins, BAPs) (Dingwall et al., 1995; Papoulas et al., 1998; Vasquez et al., 1999; Crosby et al., 1999; Kal et al., 2000). Die

oben genannten *Drosophila* Proteine BRM, MOR und SNR1 zeigen signifikante Homologien zu Proteinen in Hefe und Säugern (Tamkun et al., 1992; Dingwall et al., 1995; Papoulas et al., 1998; Crosby et al., 1999). Trotz einer Veröffentlichung, die eine physikalische Interaktion zwischen TRX und SNR1 dokumentierte (siehe unten), ist TRX nicht als feste Untereinheit des BRM-Komplexes anzusehen (Papoulas et al., 1998). Chromatin modellierende Proteinkomplexe katalysieren unter Verbrauch von ATP eine Mobilisierung der Nukleosomenorganisation *in vitro.* Auch wenn die Mitglieder der SWI/SNF Proteinfamilie weitestgehendst als transkriptionale Aktivatoren charakterisiert worden sind (Sudarnasam und Winston, 2000; Vignali et al., 2000; Narlikar et al., 2002), gibt es einige Hinweise darauf, dass manche SWI/SNF Komplexe auch an transkriptionaler Repression beteiligt sein können (Urnov und Wolffe, 2001; Martens und Winston, 2002). Das Genprodukt des trxG Gens osa reguliert die Aktivität des BRAHMA Proteinkomplexes durch Interaktion mit BRAHMA, ist aber selbst keine Unterreinheit des Komplexes (Vazquez et al., 1999). Ein weiteres trxG Gen, kismet, codiert für ein Protein, das BRM sehr ähnlich ist, aber nicht mit dem Komplex interagiert (Daubresse et al., 1999).

Die verbindende Gemeinsamkeit der ersten funktionalen trxG Untergruppe (trxG I) ist somit die Fähigkeit Chromatinstrukruren aktiv zu verändern (Chromatin remodeling).

E-3.2   TrxG II: GAGA-Faktor und ZESTE

GAGA-Faktor, codiert durch das Gen *trithorax-like* (trl), und ZESTE haben mehrere überraschend ähnliche Wirkungen. Beide sind DNA-bindende Proteine, die homeotische und andere Gene aktivieren (Biggin et al., 1988) und wirken der durch die Bildung von Heterochromatin hervorgerufenen Stillegung benachbarter DNA-Regionen (**P**osition **E**ffect **V**ariegation, PEV) entgegen, da eine Mutation beider Gene diese Effekte verstärkt (Farkas et al., 1994; Judd et al., 1995). GAGA-Faktor und ZESTE interagieren außerdem beide genetisch mit Proteinen der Polycomb-Gruppe (Phillips und Shearn, 1990; Strutt et al., 1997).

GAGA ist zudem eine der vier Untereinheiten des ATP metabolisierenden Chromatin verändernden NURF-Komplexes, der als transkriptionaler Aktivator gilt (Tsukiyama et al., 1995). ZESTE ist zudem an dem genetischen Vorgang der Transfektion beteiligt

(Pirotta, 1991). Außerdem steht es mit MOIRA im BRM Komplex in physikalischem Kontakt, um die Aktivität des Komplexes zu modulieren indem es Chromatin-Remodelling zu spezifischen Promtoren lenkt (Kal et al., 2000).

Das von Verrijzer et al. 1999 vorgeschlagene "Looping"-Model geht davon aus, dass die kooperative Bindung von GAGA-Faktor und ZESTE an multiple Zielsequenzen innerhalb eines Promotors eine Oligomerisation beider Proteine induziert, die dazu führt, dass sich die DNA des Promotors um dieses Multimer windet, und so eine normale Formation und Anordnung der Nukleosomen in diesem Promotorabschnitt verhindert. Stattdessen wird die Bindung von Transkriptionsfaktoren, welche die übliche Nukleosomen-Barriere nicht überwinden können, in diese Bereichen gefördert. GAGA und ZESTE erzeugen somit einen für Transkriptionsfaktoren leichter zugänglichen DNA-Protein-Komplex.

Proteine der zweiten funktionalen trxG Untergruppe (trxG II) teilen die Fähigkeit, die Topologie der Promotorregionen durch Reorganisation der Nukleosomenstruktur in eine für Transkriptionsfaktoren offene Region zu verwandeln und in diesem Zustand zu erhalten.

E-3.3 TrxG III: TRX, ASH1 und ASH2 als histon-modifizierende Faktoren

Die trxG Proteine ASH1 (**A**bsent **S**mall or **H**omeotic discs) (Tripoulas et al., 1994; LaJeunesse und Shearn, 1995; Tripoulas et al., 1996), ASH2 (LaJeunesse und Shearn, 1995; Adamson und Shearn, 1996) und TRX kommen in drei unterschiedlichen Proteinkomplexen mit einer Größe von jeweils 2MDa, 0.5MDa und 2MDa vor (Papoulas et al., 1998).

Der TAC1 Komplex (**T**rithorax **A**cetyltransferase **C**omplex 1) besteht aus TRX, der Antiphosphatase SBF1, und dCBP, einem Mitglied der CBP/p300 Histonacetyltransferase (HAT) Proteinfamilie. SBF1 und dCBP colokalisieren mit TRX an spezifischen Stellen auf polytänen Riesenchromosomen, wie zum Beispiel der Ultrabithorax Promotorregion, die bekannterweise von TRX reguliert wird. dCBP Mutationen erzeugen die gleichen negativen Effekte auf ubx Expression und die Expression eines lacZ Reporters, der unter Kontrolle des ubx Promoters steht, wie TRX Nullmutationen. Dies bestätigt die große funktionelle Bedeutung dieser Interaktion.

TAC1 acetyliert Histon H4 in vitro und reflektiert damit die HAT-Aktivität des homologen Säugerproteins, CBP (Petruk et al., 2001). Indem TRX die Histonacetyltransferase CBP über die TREs zu regulatorischen Abschnitten der Zielgene lenkt, kann TRX lokale Acetylierung herbeiführen und so die Bindung der Transkriptionsmaschinerie ermöglichen. Neuste Ergebnisse weisen darauf hin, dass TRX selbst eine Histonmethyltransferase (HMT) mit Spezifität für Histon H3-K4 sein könnte (Czermin et al., 2002).

Obwohl TRX und ASH1 eine weitgehend überlappende chromosomale Verteilung aufweisen, und auch aus embryonalen *Drosophila* Proteinextrakten co-immunopräzipitiert werden können (Rozovskaia et al., 1999), ist ASH1 kein Mitglied des aufgereinigten TAC1 Komplexes. ASH1 könnte somit als Vermittler zwischen dem TAC1 Komplex und spezifischen TREs dienen. Diese Möglichkeit wird durch die Beobachtung gestützt, dass der Verlust von ASH1 die Assoziation von TRX mit Chromatin zerstört. Außerdem konnte für dCBP und ASH1 eine Interaktion in vitro nachgewiesen werden (Bantignies et al., 2000). Die Gegenwart einer SET-Domäne innerhalb von ASH1 führte schließlich zu seiner Identifizierung als Histone-Methyltransferase spezifisch für H3-K4 und -K9 und für H4-K20 (Beisel et al., 2002).

Das ASH2 homologe Protein in Hefe ist Mitglied des SET1-Proteinkomplexes, der Histon H3 K4 spezifische HMT-Aktivität aufweist (Roguev et al., 2001).

Die Verbindung zu Histon H3-K4 Methyltransferaseaktivität stellt somit die Gemeinsamkeit aller Proteine in der dritten trxG Untergruppe (trxG III) dar.

Auch wenn es noch vorstellbar ist, dass im Laufe der weiteren Erforschung von trxG Proteinen auch andere Wirkweisen als Transkriptionsfaktoren zu Tage treten, so weisen alle TRX verwandten Proteine bisher eine gemeinsame Strategie auf. Alle erfüllen ihre Aufgaben durch Chromatinmodifikatoren, wie zum Beispiel durch Histon H3-K4 Methylierung und Histonacetylierung, die direkt mit aktivem Chromatin in Verbindung gebracht werden können, oder durch Chromatin-Remodelling, welches die Bindung von weiteren Transkriptionsfaktoren in transkribierten Regionen ermöglicht

E-4   Proteindomänen in TRX

Das *Drosophila* TRX Protein zeigt eine modulare Architektur und besteht aus verschiedenen selbstständigen Proteindomänen (Abbildung E-1a). Die herausragenden Domänen innerhalb TRX sind PHD-Finger und SET-Domäne.

E-4.1   Die PHD-Finger Domäne

Die **P**lant **H**omeo **D**omäne (PHD) ist ein Zink-Finger ähnliches Motiv mit einem einzigartigen Cys_4-His-Cys_3-Muster, welches sich über ca. 50 Aminosäuren erstreckt (Aasland et al., 1995) und sich so faltet, dass zwei Zn2+ Ionen auf ähnliche Weise gebunden werden können wie von einer RING-Domäne. Die Strukturanalyse enthüllt einen konservierten, Zink-bindenden Kern mit zwei variablen Bögen, welche wohl Interaktionen mit weiteren PHD-Finger Domänen und deren Liganden vermitteln (Pascual et al., 2000). Mögliche Funktionen von PHD-Fingern beinhalten DNA-Bindung, Protein-Protein-Interaktion oder zielgerichtetes Binden von Histonmodifikation (Aasland et al., 1995, O'Connel, 2001; Schultz, 2001), aber eine eindeutige Funktion konnte dieser Domäne noch nicht zugeordnet werden. Außer in TRX kommen PHD-Finger in ASH1, ASH2 und in Mitgliedern der PC-Proteinfamilie, wie z.B. Polycomb-like (PCL), als auch in vielen anderen Proteinen vor. Allerdings werden für alle diese Proteine Aufgaben im Modulieren der Chromatinstruktur vermutet.

I-4.2   Die SET-Domäne

Die SET-Domäne (benannt nach den Proteinen, in denen sie zuerst beschrieben wurde: **SU**(VAR)3-9, **E**(Z) und **T**RX) ist ein 150 Aminosäuren umfassendes Motiv, welches in chromosomalen Proteinen von der Hefe bis zum Menschen auftaucht. Erstmals in trxG und PcG Familien identifiziert, sind SET-Domänen an PEV, der Stillegung telomerer sowie zentromerer Gene und möglicherweise sogar am architektonischen Aufbau der Chromosomen beteiligt (Jenuwein, 2001).

Kürzlich wurde festgestellt, dass SET-Domänen Histonmethyltransferase (HMT)-Aktivität besitzen. Humane Proteine wie SUV39H, G9A, SET7, ESET und SET7/9, Hefeproteine wie CLR4, SET1 und SET2 und *Drosophila*-Proteine wie E(Z), SU(VAR)3-9 und ASH1 haben die Fähigkeit, Histone an ihren Lysin-Resten zu methylieren. Im Histon H3 werden Lysine an folgenden Positionen methyliert 4, 9, 27
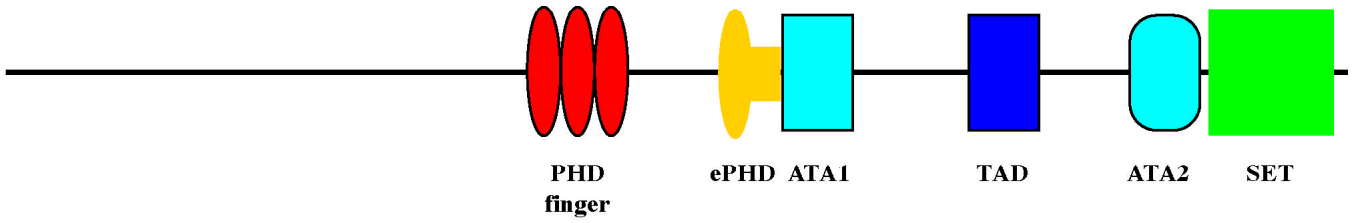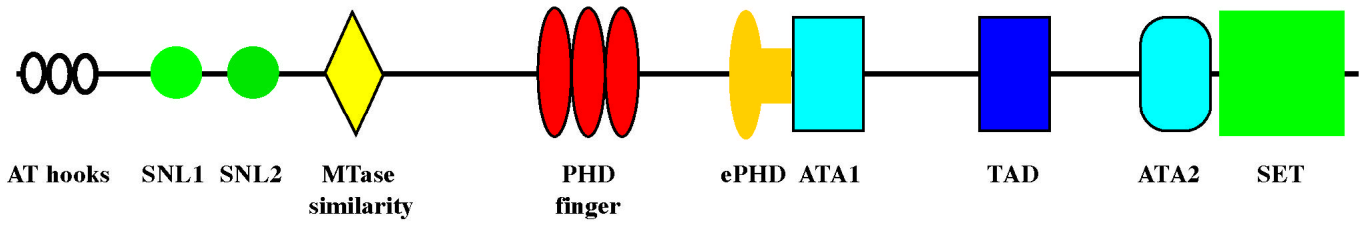
Abbildung E-1:   Proteindomaenen in *Drosophila* **TRX and Saeuger TRX2.**
(a) Das TRX Protein in Drosophila melanogaster.   **(b) Das** TRX2 Protein in Saeugern (Fuer eine detaillierte Beschreibung, siehe Text)
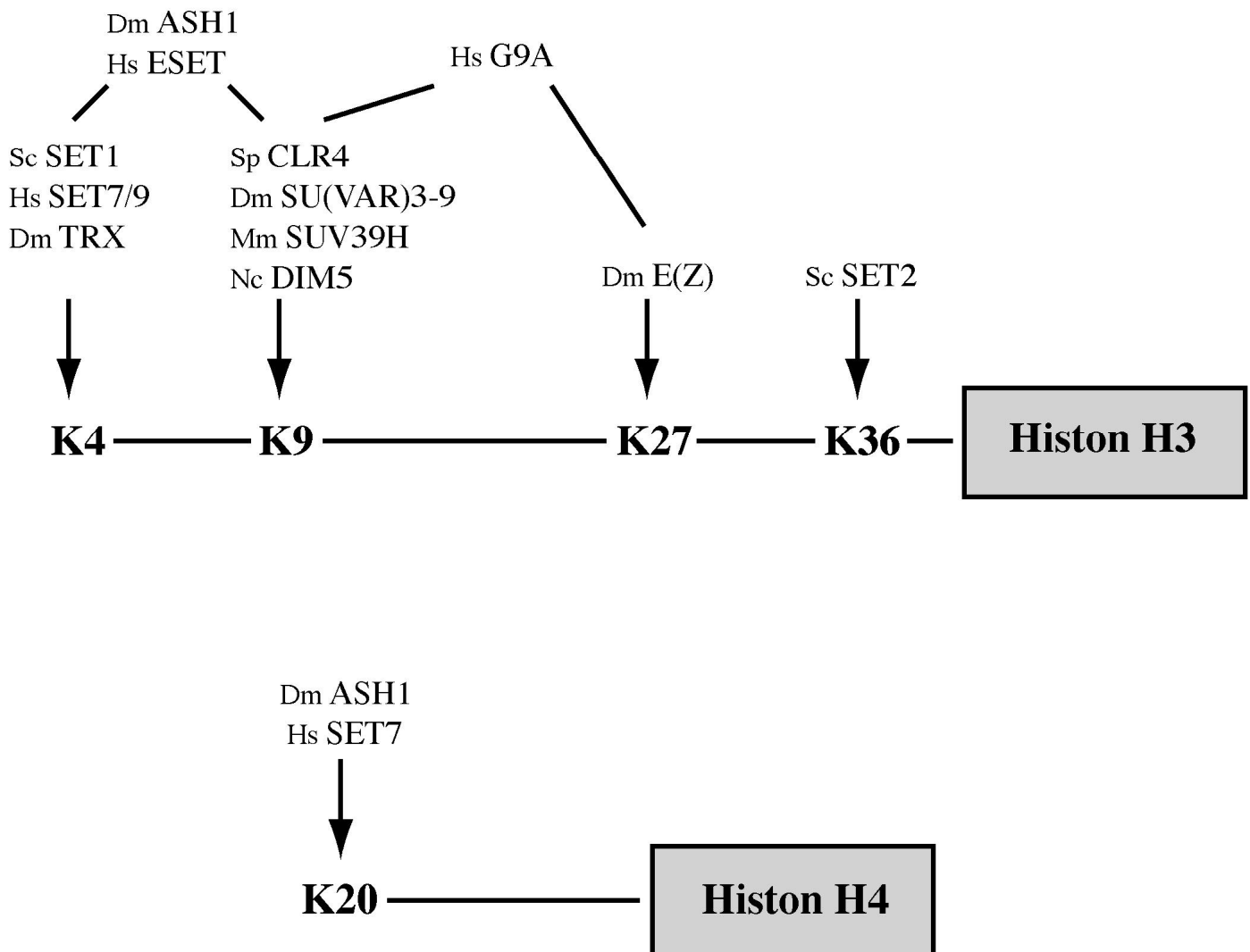
Abbildung E-2: Histon Lysin Methylierung.

Uebersicht ueber bisher bekannte Histon-Methyltransferasen und deren Lysinrestspezifitaet in Histon H3 und Histon H4 (veraendert aus Jenuwein and Lachner, 2002).

und 36, für Histon H4 ist bisher nur Position 20 bekannt. Obwohl die meisten HMTs Positionsspezifität für nur einen der genannten Aminosäurereste aufweisen, methylieren manche mehrere unterschiedliche (Lachner and Jenuwein, 2002, siehe auch Abb. E-2). Während regionale H3-K27 Methylierung in transkriptionel stillgelegtem Euchromatin (Nielson et al., 2001; Vandel et al., 2001) und in konstitutivem (Jenuwein and Allis, 2001) und fakultativem Heterochromatin (Mermoud et al., 2002; Peters et al., 2002; Boggs et al., 2002) vorkommt, zeigt H3-K4 Methylierung eine gegensätzliche Verteilung und korreliert mit aktiven Chromatinabschnitten (Litt et al., 2001). Sie existiert auch in den transkriptional aktiven Makronuclei von *Tetrahymena* (Strahl et al., 1999). Nach mehreren erfolglosen Versuchen mit rekombinantem TRX (Rea et al., 2000), konnte jetzt auch für die SET Domäne von TRX, H3-K4 HMT-Aktivität nachgewiesen werden (Czermin et al., 2002).

Die Publikation der SET-Domänen-Kristallstruktur von SET7/9 (Wilson et al., 2002) und die Sequenzanalyse der SET-Domäne N-terminal angelagerten Sequenz (Roguev et al., 2001), machten auf eine besondere Bedeutung dieser preSET-Region aufmerksam. Durch Bildung einer hydrophoben Rinne ermöglicht sie die Bindung der basischen Histon-Aminosäurenseitenketten und positioniert so das spezifische Lysin direkt im katalytischem Zentrum der SET-Domäne. In TRX wird die preSET-Region ATA2 genannt, es sind aber mindestens vier weitere preSET-Varianten bekannt (Roguev et al., 2001).

Neben der HMT-Aktivität als charakteristisches Merkmal, wurden weitere Funktionen für die TRX-SET-Domäne ermittelt. So verleiht beispielsweise die SET-Domäne von TRX die Fähigkeit zur Homodimerisierung (Rozowskaia et al., 2000), was durch die mögliche Verbindung simultan auf unterschiedliche Regionen wirkender TRX Proteine dazu führen kann, die Aktivität von gemeinsamen Zielgene zu integrieren. Auch TRX Bindung von H3, welche durch reprimierende Chromatinveränderung negativ, durch aktivierende Chromatinveränderung jedoch positiv beeinflußt wird, wird der SET Domäne zugeschrieben (Katsani et al., 2001). Die TRX-SET-Domäne kann auch mit ASH1 (Rozovskaia et al., 1999, Roguev et al., 2001) und dem *Drosophila* Homolog des Hefeproteins SNF5, SNR 1 interagieren, welches als Mitglied des Chromatin-Remodelling Komplex SWI/SNF gilt (Rozenblat-Rozen et al., 1998).

E.4.3 Die TAD-Domäne

Die TRX Transaktivierungs-Domäne (TAD) liegt im C-terminalen Teil von TRX, zwischen ATA1 (der bisher keine Funktion zugeschrieben werden konnte) und der ATA2 Region. Die TAD Domäne vermittelt die TRX-Bindung zu dCBP (Ernst et al., 2001) innerhalb des 1MDa großen Histonacetyltransferase (HAT)-Komplexes TAC1. TAC1 führt H4 spezifische Acetylierung durch und stellt die korrekte Expression von Ubx *in vivo* sicher (Petruk et al., 2001). ASH1 kann ebenfalls CBP binden, allerdings durch N-terminale Sequenzen und die SET-Domäne (Bantignies et al., 2000).

E-5 Gegensätzliche Wirkweisen von PcG und trxG Proteinkomplexen

Sowohl trxG als auch PcG Proteine wirken durch Veränderung der Chromatinstruktur. Pc-G Proteine sorgen für die vererbbare Stillegung ihrer Zielgene, indem sie Heterochromatinbildung veranlassen. Die reprimierende Wirkung von PcG Proteinen erfolgt durch Histondeacetylierung (ESC/E(Z)) (Tie et al., 2001; van der Vlag und Otte, 1999), H3-K27 Methylierung (*Drosophila* E(Z)) (Muller et al., 2002) und die Inhibition von Chromatin-Remodelling durch Behinderung der Nukleosomenerkennung des SWI/SNF Komplexes als einen nicht-katalytischen Mechanismus (PRC1) (Francis et al., 2001, Shao et al., 1999). Durch die Entdeckung, dass das PcG Protein **E**nhancer of **Z**este (E(Z)) als H3-K27 HMT wirkt (Müller et al., 2002; Czermin et al., 2002), kommt Licht in das Dunkel der trxG/PcG-Wirkweisen. Die CHROMO Domäne von Polycomb (PC, Untereinheit von PRC) selbst bindet die Methylgruppe am H3-K27 Aminosäurerest und schafft so eine direkte Verbindung zwischen dem repressiven Effekt von PRC1und der durch PcG vermittelten Methylierung (Cao et al., 2002).

Die Präsenz sich ähnelnder Proteinmodule innerhalb der PcG und trxG deutet darauf hin, dass sowohl positive als auch negative Regulationsmechanismen dieser Faktoren auf die Expression homeotischer Gene Gemeinsamkeiten aufweisen. Aufgrund der Details, die über TRX und TrxG Proteine vorliegen und hier zusammengefaßt sind, könnten TrxG Proteine den repressiven Effekten von PcG Proteinen direkt durch aktivierende Prozesse, wie Chromatin-Remodelling (BRM Komplex), H3K4-Methylierung (trxG3) oder Histonacetylierung (TAC1 Komplex) entgegenwirken.
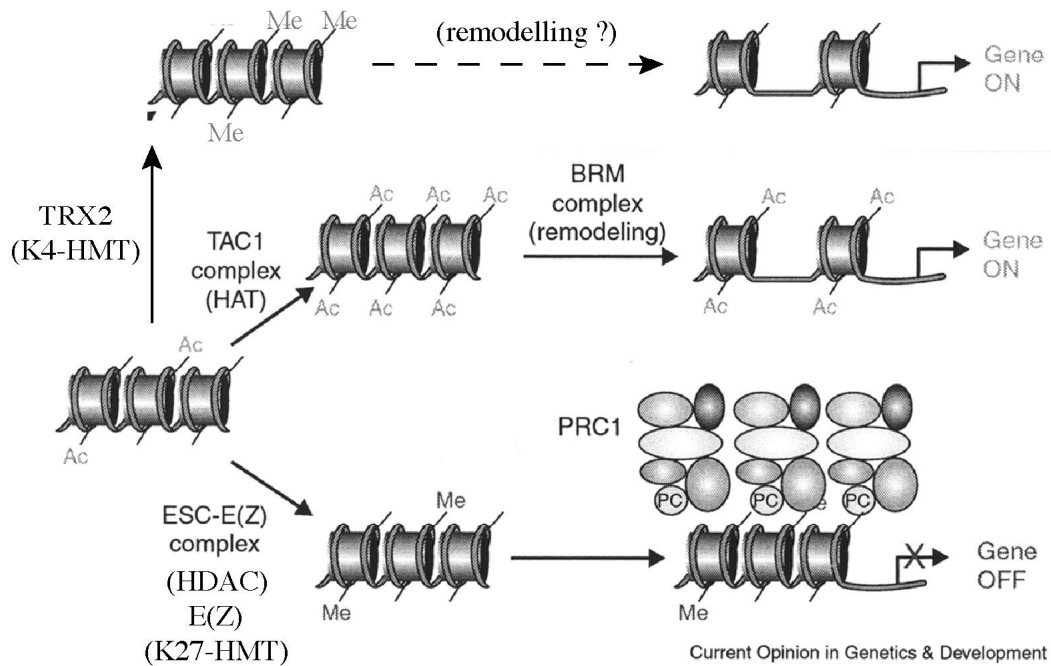
Abbildung E-3:   Modell zur Wirkungsweise der trxG and PcG Komplexe (veraendert nach Simon and Tamkun 2002)

Die schematische Darstellung bezeichnet einenNukleosomenabschnitteines Zielgens unter der Kontroll von trxG und PcG. Die zwei durch trxG hervorgerufenen aktiveirenden Prozesse activating pathways sind im oberen, der alternative PcG Repressionsweg im unteren Teil gezeigt. Ac = Acetylierung von Hitonen. Me = Methylieruing von Histonen. Bestimmte Histon-Kodierungen, die im ersten Schritt hervorgerufen wurden, helfen dann Nukleosomen veraendernde Komplexe (trxG Weg) or PRC1 (PcG Weg), zu rekrutieren, deren Wirkungsweisen den jeweils angezeigten Genexpressionszustand nach sich ziehen. Vertikale pinkfarbene Symbolezeigen entgegengesetzte Effekte der Komplexe an. Fuer eine detailliertere Diskussion, siehe Text).

Ein aktuelles Modell zur gegensätzlichen Wirkweise von trxG und PcG Komplexen ist in Abbildung E-3 dargestellt (nach Simon und Tamkun, 2002). Die nukleosom-modifizierenden Wirkungen dieser Komplexe sind dort mit dem von Jenuwein und Allis, 2001 vorgeschlagene Histon-Code verarbeitet. Der erste Schritt der durch trxG vermittelten Genaktivierung stellt Histonacetylierung durch TAC1 dar (Petruk et al., 2001). Durch den erhöhten Acetylierungsstatus wird der Brahma Komplex rekrutiert (Erleichterte Bindung von SWI/SNF and acetylierte Nukleosomen wurde schon von Hassan et al., 1999 berichtet. Die Bromo Domäne innerhalb BRMs als Modul, für das Bindespezifität für acetylierte Histone nachgewiesen wurde, könnte diese Bindung vermitteln (Dhalluin et al., 1999; Zheng and Zhou, 2002). Ein so iniziiertes Chromatin-Remodelling führt dazu, dass die DNA leichter zugänglich für Transkriptionsfaktoren wird, welche für die komplette Aktivierung nötig sind (Peterson and Workman, 2000; Vignali et al., 2000). Ein weiterer Weg wie Aktivierung durch trxG Proteine erreicht werden kann ist ebenfalls in Abbildung I-3 dargestellt: H3-K4 spezifische HMT Aktivität führt zu einem veränderten Histon-Code. Diese Markierung könnte von ISWI, dem ersten identifizierten H3-K4 bindenden Protein (Kouzarides unveröffentlichte Ergebnisse) erkannt werden. Auch wenn diese Bindung nicht für das Gegenstück in Saugern BRM gezeigt wurde, liegt die Vermutung nahe, dass auch in diesem alternativen Weg die Rekrutierung von Proteinkomplexen zur Modifizierung der Nukleosomen ein wichtige Rolle spielt.

Der erste Schritt auf dem Weg, der PcG Proteine zur Stillegung verschiedener Gene erfolgt durch Histondeacetylierung via ESC/E(Z) (v.d. Vlag et al., 1999; Tie et al., 2001) und H3-K27 Methylierung via E(Z) (Müller et al., 2002; Czermin et al., 2002). Methylierung von H3-K27 erleichtert die Bindung von PRC1 (Rastelli et al., 1993; Cao et al., 2002), eventuell vermittelt durch die Polycomb CHROMO Domäne (Messmer et al., 1992). Durch die Bildung solch repressiver PRC1 Komplexe wird anderen modifizierenden Komplexen der Zugang verwehrt (Shao et al., 1999; Francis et al., 2001) und die betroffene DNA Region wird in einem inaktiven Zustand (Heterochromatin) gehalten.

DIE TRX-G IN SÄUGERN

Alle *Drosophila* trxG Proteine haben mindestens ein Gegenstück in Saugern. Im Säugergenom gibt es zwei Homologe zum *Drosophila* trxG Gen Brahma und auch multiple homologe und analoge Proteine zu ash-l and ash2 (Nakamura et al., 2000; Huntsman et al., 1999). Trx selbst hat zwei Homologe in Säugern, Mll (auch All-1, HRX, HTRX) und Trx2 (auch Mll2, Mll4). Die Ähnlichkeiten in ihrer Aminosäuresequenz und in der Exon-Intron Struktur zeigen deutlich, das es sich bei diesen beiden Formen um paraloge Gene handelt (FitzGerald and Diaz, 1999). Die Genduplikation, welche für die beiden homologen Trx Kopien in Säugern verantwortlich war, fand evolutiv gesehen offenbar nach der Abspaltung der Vertebraten von den Invertebraten statt. Ob diese Duplikation auch mit der Vervielfältigung der homeotischen Genkomplexe zusammenfiel, von denen es in den meisten Vertebraten vier paraloge Gruppen gibt, wird bisher noch diskutiert.

Im Vergleich zum *Drosophila* trx Gen zeigen sich mehrere konservierte Bereiche, zu denen auch die PHD-Finger und die C-terminale SET-Domäne gehören. Abgesehen von diesen Sequenzen gibt es mehrere Proteindomänen, die ausschließlich in den Säugerproteinen vorkommen (figure E-1b). Sowohl MLL als auch TRX2 haben drei AT-hooks, Domänen, die sub-nukleare Lokalisation festlegen und auch eine Domäne, die sie mit der DNA Methyltransferase 1 (DNMT1) haben.

E-6   Proteindomänen exklusiv für Säuger TrxG

E-6.1   AT-hooks

Diese Domäne ist ein Sequenzmotiv, dass aus 11 konservierten Aminosäuren besteht und in der Lage ist, die kleine Rinne der AT-reichen B-Form der DNA zu binden. Erstmals wurde diese Domäne in chromosomalen Proteinen der **H**igh **M**obility **G**roup (HMG) I beschrieben (Huth et al., 1997; Reeves und Nissen, 1990). Ein Asx Bogen und kationische R/K Borsten, die lateral aus der planaren Architektur des Peptids hervorragen, tragen zur Stärkung der DNA Bindung bei. Die DNA Bindefähigkeit der AT-hooks gründet sich eher auf Struktureigenschaften, als auf spezielle Sequenzeigenschaften.

E-6.2 Die NTS Domäne

Durch Expression von kleinen MLL Teilstücken in COS-Zellen konnten **N**uclear **T**ransfer **S**ignals (NTS1 und NTS3), und zwei Sequenzabschnitte, die wichtig für eine punktförmige nukleare Verteilung (**S**peckled **N**uclear **L**ocalisation; SNL1 and SNL2) sind, gefunden werden (Yano et al., 1997). Diese punktförmige Verteilung wird auf die Assoziation von MLL mit Komponenten der Kernmatrix zurückgeführt, wodurch ihm eine Rolle in der Modulation der Chromosomenstruktur zugewiesen wurde, welche zur epigenetischen Erhaltung der Genexpression führt (Caslini et al., 2000).

E-6.3 Die MT-Domäne

Die Existent der etwa 100 Aminosäuren umfassenden MT-Domäne in MLL, wurde erstmalig 1993 von Ma et al. entdeckt. Außer in DNMT1 kommt dieses Motiv auch im Transkriptionsrepressor MeCP1 (Cross et al., 1997), dem methyl-CpG bindenden Protein MBD1 (Fujita et al. 1999 and 2000) und dem CpG-bindenden Protein hCGBP (Voo et al., 2000) vor. Es setzt sich aus einem Cystein-reichen Abschnitt zusammen, der von basischen Aminosäuren umgeben ist. Der Cystein-reiche Abschnitt zeichnet sich durch zwei Kopien der Aminosäurensignatur CGxCxxC aus (wird daher auch CxxC Domäne genannt) und ermöglicht die Unterscheidung von methylierter, hemimethylierter und unnmethyliereter DNA (Birke et al., 2002).

Innerhalb von MLL ist die MT-Domäne in eine größere Region eingelagert, die als Repressor-Domäne bezeichnet wird, weil sie, durch Fusion an die GAL4 DNA-Bindedomäne und artifizielle Rekrutierung an einen Promotor, Transkription repremieren kann (Zeleznik-Lee et al., 1994; Birke et al., 2002). GAL4 Fusionsproteine mit der MT-Domäne von DNMT1 zeigen ebenfalls repremierende Wirkung (Fuks et al., 2000). Im Falle der DNMT1 MT-Domäne ist die Repression mit Histondeacetylierung verknüpft, da erstens die durch das Fusionsprotein vermittelte Repression durch einen HDAC Inhibitor, Trichostatin A (TSA), aufgehoben wurde, aber auch weil die beiden mittlerweile aufgereinigten DNMT1 Proteinkomplexe HDACI, und HDACII respektive, beinhalten (Robertson et al., 2000; Rountree et al., 2000).

Das Vorkommen dieser MT-Domäne deutet auf eine Verbindung zwischen der TRX2/MLL Wirkungsweise und dem Methylierungsstatus des Genoms hin. Mit Hilfe der

MT-Domäne könnten TRX2 und MLL die Grenzen zwischen aktivem und inaktivem Chromatin aufspüren, die durch eine Veränderung in DNA Methylierungsstatus gekennzeichnet sind. In Analogie zu DNMT1 könnten sie dort Proteinkomplexe rekrutieren, und so die Weitergabe von epigenetischen Signalen über Generationen hinweg sichern.

Alle erwähnten Domänen sind eigenständig, was darauf hinweist, dass TRX2 verschiedene Chromatin modulierende Funktionen in Form von modularen Proteindomänen vereinigt. Die Kombination von AT-hooks, SNL-, NTS- und MT-Domänen deutet darauf hin, dass das Säuger TRX hauptsächlich auf Response-Elemente wirkt, die AT-reiche Abschnitte und unmethylierte CpG Inseln umfassen. Dies sind auch die Charakteristika von aktiven Promotorregionen. Somit könnten MLL und TRX2 die Aufrechterhaltung der Aktivität von vorweg aktivierten Promotoren durch die chromatinmodifizierenden Mechanismen, wie sie für ihr Drosophila Homolog beschrieben sind, gewährleisten.

E-7   MLL und seine Bedeutung fuer menschliche Tumoren

Das 400kDa große Säugerprotein MLL (**M**ixed **L**ineage **L**eukemia) zeigt im Verlauf der embryonalen Entwicklung eine ubiquitäre Expression. Das humane Mll Gen liegt auf Chromosom 11 in der Region 11q23 und spielt durch chromosomale Translokationen in verschiedenen kindlichen myeloiden und lymphoiden Leukämieerkrankungen eine Rolle (Rowley, 1993; Bernard und Berger, 1995; Rubnitz et al., 1996; Look, 1997; Gilliland, 1998). Diese Verbindung führte zu der Annahme, dass MLL in seiner nativen Form eine wichtige Rolle in der frühen Entwicklung des blutbildenden Systems spielt (Ernst et al., 2002). Die Region, die an der Translokation beteiligt ist liegt nahe bei Intron 11 und ist klar eingegrenzt; man nennt sie BCR (break point cluster region). In Translokationen fusioniert der N-terminale Teil MLLs mit einer Vielzahl von Fusionspartnern (DiMartino and Cleary, 1999). Da die Fusionspartner keine Gemeinsamkeiten aufweisen, bleibt ihre Rolle beim krebserregenden Effekt dieser Translokationen unklar. Es scheint, dass die Kombination zweier Faktoren für die von MLL hervorgerufene Tumorgenese

verantwortlich ist. Erstens könnte das Abtrennen des C-terminalen Teils zu einer dominant-negativen MLL-Form führen (Prasad et al., 1994; Schichmann et al., 1994, 1995), was zweitens durch die Addition des Fusionspartners noch verstärkt wird. Das Leukämie hervorrufende Fusionsprotein behält weiterhin die AT-hooks und die MT-Domäne, aber nicht mehr die PHD-Finger und die SET Domäne von MLL. Durch den Fusionspartner wird die Stabilität des Fusionsproteins und /oder eine Plattform für die MLL-Dimerisierung geschaffen (Dobson et al., 2000).

E-7.1 Mausmodelle mit mutiertem Mll Gen

Durch die phänotypische Analyse von Mäusen, in denen das Mll Gen mutiert wurde, wird die Hypothese, dass MLL in Säugern eine ähnliche regulatorische Funktion erfüllt wie das *Drosophila* Protein TRX, bestärkt. Bisher wurden drei unterschiedliche Knock-Out Strategien für das Mll Gen publiziert:

In der ersten Veröffentlichung von Yu et al.,1995 wurde MLL durch das Einfügen einer lacZ-polyA-Kassette in Exon 3 unterbrochen. Diese Strategie führte zur Produktion eines stark verkürzten Mll-Transkripts, welches direkt hinter dem AT-hook endete. Heterozygote Tiere waren lebensfähig, zeigten aber einen komplexen Phänotyp, was darauf hindeutet, dass Mll haploinsuffizient ist. +/- Mäusenachkommen waren kleiner als Wildtyptiere und wiesen eine weibliche Hypofertilität, Anämie und eine geringere B-Zell Population auf. Segmentale Abnormalitäten traten mit unvollständiger Penetranz auf, und schlossen Skelettmissbildungen und bidirektionale homeotische Transformationen ein. Sowohl anteriore Transformationen der cervicalen Wirbel C7 nach C6 und Brustwirbel T3 nach T2 als auch posteriore Transformationen von T13 nach Lendenwirbel L1 und L6 nach Sakralwirbel S1 waren zu beobachten. Auf molekularer Ebene konnten per RNA *in situ* Hybridisierung posteriore Verschiebungen der hox-c9 und hox-a7 Expressionsgrenzen festgestellt werden. Bidirektionale Transformationen als auch caudal verschobene Expression der homeotischen Gene spiegeln den homeotischen Phänotyp des *Drosophila* trx Knock-Outs wieder. Embryonale Lethalität wurde ab E10.5 für homozygote Tiere ermittelt. Während das spatiotemporale Anschalten von hox-c9 und hox-a7 gegen E8.5 normal verlief, so konnte ab E9.0 keinerlei Genexpression mehr

verzeichnet werde (Yu et al., 1998). Der Verlust der Fähigkeit, die Expression dieser Gene aufrecht zu erhalten, ist ebenfalls analog zu den in Fliegen beobachteten Effekten.

Im zweiten Mll Allel (Yagi et al., 1998) wurde die Transkription nach Exon 11 unterbrochen, was in +/- Tieren außer zu einer leichten Anämie nicht zu einem Phänotyp führte. -/- Embryos dagegen starben zwischen dem Entwicklungstag E11.5 und E14.5, wahrscheinlich aufgrund von Ödemen. Dies weist auf eine Beteiligung MLLs an der embryonalen Blutentwicklung hin. Es wurde vorgeschlagen, dass verringerte Hox-Gen-Expression die Differenzierung hematopoetischer Vorläufer in Mitleidenschaft gezogen haben könnte, um so den erwähnten Phänotyp hervorzurufen. Der Grund für die Verschiebung des Lethalitätszeitpunkt im Vergleich zum ersten Mll Allel könnte in der unterschiedlichen Knock-Out-Strategie zu suchen sein: Der Austausch der Exons 12-14 durch einen Neomycin Selektionsmarker, führte zur Expression eines N-terminalen Transkripts, inklusive der AT-hooks und der MT-Domäne und könnte somit noch einige lebensnotwendige Aufgaben von MLL erfüllen. Somit entsteht durch diese Strategie ein hypomorphes Allel, welches verzögerte embryonale Lethalität induziert.

Ayton et al. veröffentlichten 2001 das dritte Mll Allel. In diesem Fall wurde Mll durch das Einfügen einer STOP-IRES-lacZ Kassette hinter das fünfte Exon unterbrochen. In heterozygoten Tieren führte dies nur zu milden Skelettdefekten, doch die homozygoten Embryonen entwickelten sich nie weiter als bis zum 2-Zell Stadium. Der Unterschied in der Schwere des Phänotyps verglichen mit den beiden vorangegangenen Allelen wird weiterhin diskutiert, doch könnte er damit erklärt werden, dass das dritte Allel einen dominant negativen Effekt hat. Im Gegensatz zum ersten Allel, produzierten Ayton et al. ein Transkript, das sowohl AT-hooks, als auch die MT-Domäne beinhaltet. Beide Domänen binden bekannterweise DNA nicht in einer sequenzspezifischen, sondern einer strukturabhängigen Weise, so dass die Entstehung eines neuen dominant negativen Repressors vorstellbar ist. Allerdings ist das dritte Allel auch das Einzige, das nicht zu einem Fusionsprotein zwischen dem unterbrochenen MLL und LacZ bzw. Neomycin führt. Diese Fusion könnte wie im Falle der echten Translokationsprodukte bei Leukämien zur Stabilisierung des verkürzten Protein führen. Auch unsere unpublizierten Ergebnisse mit einem weiteren, vierten Mll Allel weisen daraufhin, dass es besonders schwierig ist, Mll -/- Embryonen zu finden. Somit könnte das Mll Allel von Ayton et al.

tatsächlich den wirklichen Verlust MLLs widerspiegeln. Es muß aber erwähnt werden, dass in keiner der drei Publikationen, das Vorkommen eines verkürzten Restproteins erfolgreich untersucht wurde.

Als Zielgene für MLL konnten die hox Genkomplexe hox-A und hox-C identifiziert werden: Yu et al. zeigten, dass hox-a7 und hox-c9 Expression durch den Verlust von Mll im gesamten Embryo beeinflußt war. Yagi et al. fanden verschobene Expressionsgrenzen der Gene hox-a7, -c8 und -c9 in unfraktionierten embryonalen Leberzellen von -/-Embryos. Hanson et al. untersuchten 1999 die Expression der Hox Gene in E10.5 **M**ouse **E**mbryonic **F**ibloblasts (MEFs) und stellten fest, dass hox-c4 bis -c9 und hox-a3 bis -a10 entweder gar nicht mehr oder stark reduziert in Mll-/- Zellen expremiert werden. Interessanterweise scheint in keiner der Untersuchungen die Expression der beiden anderen hox Genkomplexe, hox-B und hox-D, in Mitleidenschaft gezogen zu sein.

E-8   TRX2

Das Mll-Schwestergen, Trx2, liegt auf Chromosom 19 (19q13.1) und erstreckt sich über eine genomische Region von 35kb. Die 8.5 kb große mRNA besteht aus 37 Exons, weist die gleiche Exon-Intron Struktur wie Mll auf, und wird in ein 280 kDa (2715 Aminosäuren) großes Protein transplantiert. Da heterozygote Mll Mutanten einen dokumentierten Phänotyp aufweisen (siehe oben), kann davon ausgegangen werden, dass TRX2 die Mll Mutation nicht kompensieren kann. Dies deutet daraufhin, dass die Wirkweisen der beiden Proteine zumindest nicht vollständig überlappen.

E-8.1 Beteiligung von TRX2 an humanen Tumoren

Das Trx2 Gen liegt in einer Region, die in soliden humanen Tumoren häufig rearrangiert oder vervielfältigt ist (Mitelman et al., 1997). Die deutliche Ähnlichkeit zwischen Mll und Trx2 im Bezug auf die beibehaltene Exon-Intron Struktur des Gens, auf den modularen Aufbau aus den gleichen Domänen und die Tatsache, dass Trx2 ebenfalls in einer Region häufiger Rearrangierung liegt, weisen auf eine mögliche Beteiligung von TRX2 in der Tumorgenese hin. Tatsächlich konnte eine Vervielfältigung von Trx2 in zwei Pankreastumor-Zellinien und einer Glioblastoma-Zellinie festgestellt werden

(Huntsman et al., 1999). Schon Experimente in Drosophila zeigten, das die Dosierung der trx Expression wichtig für die kontrollierte Hox Genexpression ist (Gould et al., 1990).

E-8.2 Mausmodelle mit mutiertem Trx2 Gen

Stefan Glaser im Labor von Prof. A.F. Stewart analysiert zur Zeit ein Trx2 Knock-Out Mausmodell. Das mutierte Trx2 Gen wird in diesem Fall nach Exon1 unterbrochen. (siehe auch Ergebnisteil). Heterozygote Tiere sind lebensfähig, fertil und zeigen keinen vom Wildtyp abweichenden Phänotyp. Die homozygote Mutation führt zu embryonaler Lethalität am Entwicklungstag E10.5 und zeigt ein Wachstumsdefizit, das erstmals am E7.5 zu beobachten ist (figure E-4). Die Gastrulation als einer der Eckpunkte der embryonalen Entwicklung, die zur Bildung der drei embryonalen Keimblätter (Ekto-, Meso- und Endoderm) führt, findet zwischen E6.5 und E7.0 statt. Der sich im embryonalen Ektoderm anschließende Vorgang der Neurulation (E7.5-8.0), bildet die Neuralplatte, die letztendlich zur Entwicklung des Gehirns und des Neuralrohrs führt. Der Zeitpunkt E7.5 in der embryonalen Entwicklung ist auch durch die Bildung extraembryonaler Gebilde gekennzeichnet, die eine wichtige unterstützende Rolle für den heranwachsenden Embryo spielen. Organogenese beginnt um den Entwicklungszeitpunkkt E8.5; hier wird die Entwicklung aller wichtigen Organsysteme initiiert. Aus der Tatsache, dass sich der Trx2 Phänotyp erstmals bei E7.5 abzeichnet, läßt sich möglicherweise schließen, dass die Embryonen die Gastrulationsphase normal und ohne Wachstumseinschränkungen durchlaufen. Dennoch führen entweder eine Störung in der Segmentfestlegung während der Gastrulation, oder die verhinderte Durchführung anderer wichtiger morphologischer Umwandlungen, die in den darauffolgenden Entwicklungsabschnitten (Neurulation oder Organogenese) durchlaufen werden, zu einem eingeschränkten Wachstum und letztendlich zur Resorption des mutierten Embryos. TRX2 scheint eine Vielzahl von Auswirkungen auf die essentiellen Entwicklungen in diesen Stadien zu haben.

Nicht verifizierte und unveröffentlichte Studien mit Hilfe von RNA in situ Hybridisierung in unserer Arbeitsgruppe konnten zwei mögliche Trx2 Zielgene ermitteln, Otx2 und Hox-b1. Otx2 spielt eine entscheidende Rolle in der Gehirnentwicklung und hox-b1 ist Mitglied de Hox-B Genkomplexes. Dies weist darauf hin, dass Trx2 ebenso
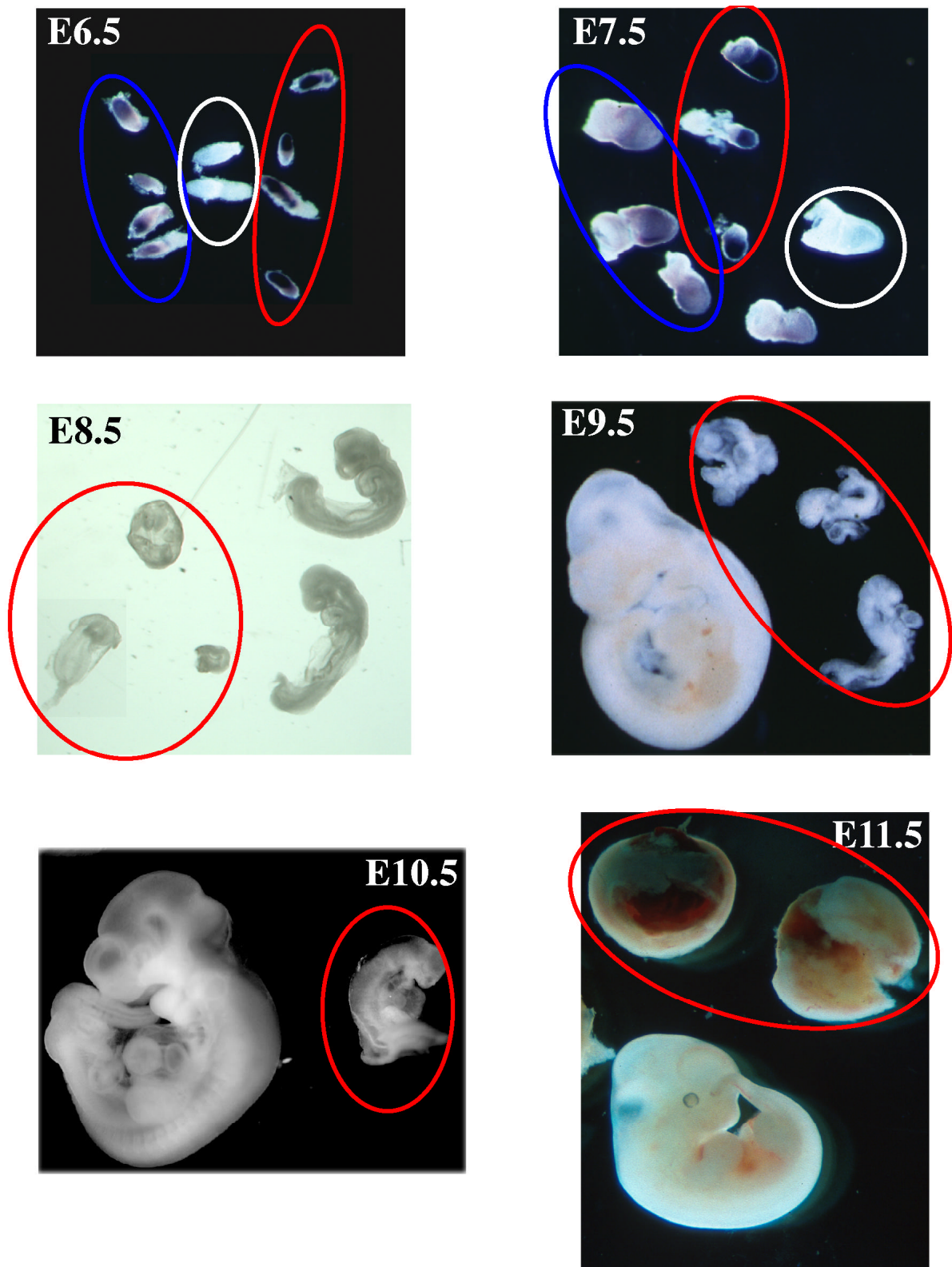
Abbildung E-4:   Phenotyp der Trx2 knock-out Embryonen.

Fuer das Entwicklungsstadium E6.5 and E7.5 sind lacZ Faerbungen des ganzen Embryos gezeigt. Dank der Eigenschaften der k.o. Kassette, kann das Trx2 k.o. Allel durch lacZ Expression sichtbar gemacht werden. Wt Embryonen sind weiss (angedeutet durch ein weisses Oval) waehrend heterozygote (angedeutet durch ein blaues Oval) and homozygote (angedeutet durch ein rotes Oval) Embryonen durch unterschiedlich starke Blaufaerbung unterschieden werden koennen. Fuer Studie spaeterer Stadien sind ungefaerbte Embryonen gezeigt. Beide, wt und +/- Embryonen wt Groesse, waehrend -/- Embryonen retardiert sind. Vor E7.5 zeigen Trx2-/- Embryonen keinen abnormalen Phaenotyp. Ab E7.5 sind -/- Embryonen in Groesse und Entwicklungsstadium mit unterschiedlicher Penetranz retardiert, verglichen mit ihren wt Geschwistern. Nach E10.5 werden keine -/- Embryonen gefunden. Die hoch retardierten rot umrandeten -/- Embryonen von E11.5 sind im Prozess der Resorbtion.

wie sein Gegenstück in *Drosophila* und sein Säugerortholog MLL an der Regulation homeotischer Gene beteiligt ist. Interessanterweise ist bisher noch kein Mitglied des Hox-B oder de Hox-D Genkomplexes als Zielgen für MLL beschrieben worden. Dies könnte zu der Schlußfolgerung führen, dass TRX2 und sein nahe verwandtes Protein MLL in der Regulation der vier Hox Genkomplexe zusammenarbeiten. MLL reguliert wie bewiesen hox-A und hox-C, während TRX2 möglicherweise die Regulation von Hox-B und Hox-D übernimmt. Die Expression der potentiellen Zielgene Otx2 und Hox-b1 wird in Trx -/- Embryonen zum richtigen Zeitpunkt initiiert, aber eine Aufrechterhaltung der Expression findet nicht statt (unsere unveröffentlichten Ergebnisse), was wiederum zu der Wirkweise des Drosophila Proteins TRX, als positiver Faktor zur Aufrechterhaltung von Genexpression, paßt.

Zusammenfassend kann man festhalten, dass die trxG Proteine in Säugern, wie auch die trxG Proteine in Fruchtfliegen, wichtige Rollen in der Aufrechterhaltung von Expressionsmustern während der Embryonalentwicklung übernehmen. Dies wird wahrscheinlich mittels aktiver oder repressiver Chromatinabschnitte erreicht.

E-9   Zielsetzung der Arbeit

Genetische Studien in *Drosophila* enthüllten und charakterisierten eine neue Art von Transkriptionsregulatoren, genannt Trithoraxgruppe (trxG). Nur wenig ist allerdings darüber bekannt, wie trxG Mitglieder in Säugetieren arbeiten. Diese Studie konzentriert sich auf eins der säugerorthologen Proteine des Gründungsmitglieds der Trithoraxgruppe, Trithorax. Bemühungen in unserem Labor, die Eigenschaften von Trx2 in Säugern zu verstehen, beschränkten sich bisher hauptsächlich auf seine frühen Funktionen während der Embryonalentwicklung, da die frühe Lethalität der Trx2 k.o. Maus Studien späterer Funktionen ausschließen. Trx2 scheint während der gesamten Embryonalentwicklung und auch im Adulten ubiquitär exprimiert zu sein. Da Studien an Drosophila-TRX implizieren, dass es möglicherweise durch epigentische Regulation eine wichtige Rolle

als Faktor zur Aufrechterhaltung der Genexpression übernimmt, war es eine unserer Prioritäten, experimentelle Wege zur Erforschung der Trx2 Wirkweisen nach der ersten embryonalen Krise, (hervorgerufen durch seinen Verlust) zu finden.

Wir wählten zwei unterschiedliche Wege, um die Beteiligung von Trx2 an Differenzierung und fötaler Entwicklung zu studieren. Eine ES Zellinie homozygot für den k.o. von Trx2 wurde benutzt, um die Auswirkungen des Verlusts von TRX2 auf Lebensfähigkeit und zelluläre Funktion während des undifferenzierten Zustandes zu beobachten. Zusätzlich sollten Studien über das in vitro Differenzierungspotential der Trx2-losen ES Zellen dazu dienen, die Rolle von Trx2 während des Differenzierungsprogrammes verschiedenster Zelllinien zu erforschen. Blastozysten-Injektionen und Analyse der chimären Embryonen boten einen Weg, die Funktion von Trx2 während später Phasen der Embryonalentwicklung auszuleuchten. Da Hinweise aus Fliegenstudien darauf hindeuten, dass Trx2 eine wichtige Bedeutung in der Chromatinregulation durch Histonmethylierung spielen, sollte auch posttranslationale Modifizierungsmuster der Trx2 -/- ES Zellen untersucht werden.

In einem zweiten Ansatz wurde durch einen knock-in in den endogenen Lokus von ES Zellen eine Fusion zwischen Trx2 und einem Fluoreszenzmarker erreicht, um die Zellbiologie von Trx2 durch ein Verfolgen der subzellulären Verteilung des Proteins vor und während verschiedener Differenzierungsstadien zu erforschen.

Summary

Drosophila trxG and PcG proteins are epigenetic regulators whose activity is required to maintain the proper expression pattern of homeotic genes during fly embryonic development. Both protein families have shown to convey their opposing effects by influencing the open or repressed states of chromatin. While some mammalian members of trx-G like Mll have already been proven to have a regulatory influence on expression of the Hox genes, the action, target genes and localization of the second mammalian homologue Trx2 remains mostly unexplored. Work in this thesis has focused on the study of mammalian TRX2 function during mouse embryonic development.

Successful generation and analysis of a knockout Trx2 ES cell line allowed us to conclude that viability and cell cycle regulation is not altered by loss of TRX2 in the totipotent state of embryonic stem cells. By an N-terminal fusion of the **E**nhanced **Y**ellow **F**luorescent **P**rotein (EYFP) to murine TRX2 we have created a tool to study TRX2 sub cellular localization in **E**mbryonic **S**tem (ES) cells. Analysis of the hypomorphic phenotype of EYFP-Trx2 homozygous mice enabled us to draw conclusions about the requirement of functional TRX2 in late phases of embryonic, fetal and adult development, an observation that is further supported by blastocyst injection experiments of Trx2-/- ES cells. Low chimeric embryos display a variable but overall contribution of Trx2 -/- cells until E10.5, but progressive elimination of knockout cells until E18.5. If the contribution of -/- cells was about a certain threshold those highly chimeric embryos reflected the phenotype of Trx2 knockout mice ensuring that the Trx2-/- phenotype is not caused by a defect in the extraembryonal tissue but in the embryo proper.

All performed experiments indicate that Trx2 is continuously required during development in a cell-type unspecific and cell-autonomous manner.

I. Introduction

For a correct determination of cell identities throughout the body axis of the developing embryo, a faultless expression of homeotic genes is required. Misregulation of members of this group of genes results in transformation of one body segment into a different one. Homeotic gene expression is established by the products of segmentation, pair-rule and gap genes, while two groups of proteins are involved in maintaining the expression patterns established by these initial regulators: TrxG and PcG. Pc-G proteins act as maintenance factors for repression, while trxG proteins are responsible for maintaining the active state of expression. Both PcG and trxG proteins are thought to function by establishing closed or open chromatin configurations at their target genes. (For reviews see Mamoudi and Verrijzer, 2001; Paro et al., 1998; Pirotta, 1998; Gould, 1997)

This work will focus on the function of the mammalian protein TRX2, which is homologous to the founding member of the trx-G in flies. Since work in this field so far has mainly been performed in the model system *Drosophila melanogaster*, the chapter will start by introducing known features about the fly homologue Trx.

TRX-G IN *DROSOPHILA MELANOGASTER*

I-1   Trx

The *D. melanogaster* trx gene encodes a large protein whose continued expression is required for proper embryonic development of the fly (Ingham 1981; 1985). Trx was first discovered by Lewis in 1968 as a regulator of bithorax (Rg-bx) and has shown to be responsible for correct transcription of the homeotic genes within both the antennapedia- and bithorax complexes (ANT-C and BX-C). Trx mutant phenotypes include homeotic transformations in which segments are transformed mainly towards more anterior identities (Breen, 1999). Most trx alleles are zygotic lethal, some weak trx alleles have third halteres develop as second thoracic wing, or male genitalia (usually located on T3) transformed to T2 leg etc. The name Trx itself comes from a hypomorphic trx allele

whose phenotype displayed bidirectional transformations of both the first and third thoracic segment into the second thoracic segment so that mutant flies were born with three thoraxes (Ingham, 1998). This bidirectional transformation is a not a common feature of all trx alleles, but is rather unusual. Still it remains as the name-giving feature of the gene.

Trx cytogenetic region spans 40 kb on the right arm of chromosome 3 (region 88B) (Mozer and Dawid, 1989; Breen and Harte, 1991). The transcription unit of 25 kb consists of 8 exons that encode for two protein isoforms TRXI of 3358 and TRXII of 3726 amino acid residues. The protein isoforms differ by 368 N-terminal amino acids that are encoded in the alternatively used exons 2 and 3 (Mazo et al., 1990; Breen and Harte, 1991; Sedkov et al., 1994; Stassen et al., 1995). At least 3 differentially spliced mRNA forms of trx exist:

- The 10 kb mRNA encoding TRXI is maternally supplied to oocytes and is only present in 0-3 hour embryos (Mozer and Dawid, 1989; Breen and Harte, 1991; Sedkov et al., 1994).

- The 12 kb mRNA also encodes TRXI and is present mainly in blastoderm embryos then in decreasing levels throughout development (Mozer and Dawid, 1989; Breen and Harte, 1991; Sedkov et al., 1994).

- TRXII is encoded by a 14 kb mRNA that is present from germband elongation through larval and pupal stages, and the only RNA expressed during imaginal cell proliferation (Mozer and Dawid, 1989; Breen and Harte, 1991; Sedkov et al., 1994).

The zygotic RNAs are expressed ubiquitously in early stages of development. In late stage embryos, trx is preferentially expressed in the CNS (Mozer et al., 1989). Westertn blot analysis showed that while TRXI is the most prevalent isoform during early stages, TRXII is predominant during the final third of embryogenesis (Kuzin et al., 1994).

I-2   TRX target genes and response elements

Consistent with the phenotypic observations for mutant trx alleles, trx target genes include members of the homeotic family of genes like *Ultrabithorax, abdominal-A, Abdominal-B, Antennapedia, Sex combs reduced* and *Deformed* (Mazo et al., 1990; Breen and Harte, 1993; Sedkov et al., 1994). Besides homeotic genes, the transcription of the transcription factors *engrailed* (Breen et al., 1995) and *fork head* (Kuzin et al., 1994) is dependent on trx function.

The way in which the Pc-G and Trx-G products bind their target genes remains still largely unknown. Even though most of the characterized PcG or trxG proteins seem not to bind DNA in a sequence dependent manner, genetic and biochemical studies have identified DNA elements responsive to those proteins (trxG and PcG response elements; TREs and PREs). TREs and PREs have been found in the regulatory region of *Ultrabithorax* and *sex combs reduced,* but do not show a simple structure with few significant DNA sequence motifs (Castelli-Gair and Garcia-Bellido, 1990; Chan et al., 1994; Chang et al., 1995; Gindhart and Kaufmann, 1995). TRE and PRE activities can be ascribed to separable DNA elements, even though they are located in very close vicinity (Tillib et al., 1999). This proximity suggests some direct interaction, but it argues against direct competition of the opposing groups for their target sites. Consistent with this observation is the finding that TRX binds to specific sites on polytene chromosomes and co-localizes with Pc at many sites (Chinwalla et al., 1995)

I-3   The trx-G of proteins and complexes

Classification of a gene as a member of the trxG has been variable and certain genetic criteria have been used:

- A gene shows a homeotic mutant phenotype similar to loss-of function mutations in homeotic genes
- Mutations in a gene enhance the phenotype of other trxG members
- Mutations in a gene suppress the dominant phenotype of PcG mutants.

The trxG is a heterogeneous group of proteins that are involved in the various regulatory steps to maintain HOM gene function. The range of action of trxG genes is varied and argues against any model in which all trxG products act together in one complex or mechanism. The trxG can be divided into at least three functional subgroups:

1. TrxG I: Brahma and the Swi/Snf connection
2. TrxG II: GAGA factor and Zeste
3. TrxG III: Trx, ash1 and ash2 as histone modifiers

I-3.1 TrxG I: Brahma and the Swi/Snf connection

The BRM complex contains the trxG proteins Brahma (BRM) and Moira (MOR), the **S**nf5 **R**elated **P**rotein SNR1 and four additional **B**rahma **A**ssociated **P**roteins (BAPs) (Dingwall et al., 1995; Papoulas et al., 1998; Vasquez et al., 1999;Crosby et al., 1999; Kal et al., 2000). The three first mentioned complex members show significant homology to counterparts in yeast and mammals as summarized in figure (Tamkun et al., 1992; Dingwall et al., 1995; Papoulas et al., 1998; Crosby et al., 1999). Despite a reported physical association between TRX and SNR1 (see below), TRX is not a subunit of the BRM complex (Papoulas et al., 1998).

Chromatin remodeling complexes catalyze ATP-dependent alterations in nucleosome organization *in vitro*. Even though the SWI/SNF family members are well characterized as transcriptional activators (Sudarnasam and Winston, 2000; Vignali et al., 2000; Narlikar et al., 2002), there are some indications that suggest SWI/SNF complexes play roles in transcriptional repression (Urnov and Wolffe, 2001; Martens and Winston, 2002). The trxG gene osa is thought to regulate activity of the BRM complex via interaction with BRM, but is not a bona fide member of the complex itself (Vazquez et al., 1999).

Another trxG gene, kismet, encodes proteins highly related to BRM, but does not physically interact with the complex (Daubresse et al., 1999).

The unifying property of trxG I members therefore is their involvement in chromatin remodelling.

I-3.2   TrxG II: GAGA factor and ZESTE

GAGA factor, encoded by the *trithorax-like* (trl) gene, and Zeste protein perform surprisingly various but similar modes of functions. Both are DNA binding proteins activating homeotic and other genes (Biggin at al., 1988). When mutated both are enhancers of PEV and therefore counteract heterochromatin induced silencing (Farkas et al., 1994; Judd et al., 1995) and show genetic interactions with PcG proteins (Phillips and Shearn, 1990; Strutt et al., 1997). GAGA factor has been found to be one of the four subunits in the ATP-dependent chromatin remodelling complex NURF, a transcriptional activator that also alters nucleosome structure (Tsukiyama et al., 1995). Zeste additionally is involved in transvection (Pirotta, 1991) and contacts Moira in the BRM complex to direct chromatin remodeling to specific promoters and modulate its activity (Kal et al., 2000).

The model in Verrijzer et al. 1999 for co-operative GAGA binding suggests that binding of GAGA factor and ZESTE to multiple target sites within a promoter induces oligomerization of the proteins and wrapping of the promoter DNA around that multimer in a way that excludes normal nucleosome formation. Instead it allows binding of transcription factors that are unable to overcome the nucleosome barrier by themselves. GAGA and ZESTE therefore serve as factors that provide a more accessible DNA-protein complex.

The trxG II is unified by this looping model, in which promoter topology reorganization leads to the maintenance of an open chromatin conformation.

I-3.3   TrxG III: TRX, ASH1 and ASH2 as histone modifiers

The trxG proteins **A**bsent **S**mall or **H**omeotic discs (ASH1) (Tripoulas et al., 1994; LaJeunesse and Shearn, 1995; Tripoulas et al., 1996), ASH2 (LaJeunesse and Shearn, 1995; Adamson and Shearn, 1996) and TRX (see above) are found in three distinct protein complexes of 2 MDa, 0.5 MDa and 2 MDa size respectively (Papoulas et al., 1998).

The TRX complex called TAC1 (for **T**rithorax **A**cetyltransferase **C**omplex 1) contains the antiphosphatase SBF1 and a member of the CBP/p300 family of histone acetylltrasferases (HATs) called dCBP. SBF1 and dCBP colocalize with TRX at discrete

sites on polytene chromosomes including at Ubx known to be regulated by TRX. Mutations in dCBP cause the same negative effects as null trx mutations on Ubx expression and the expression of lacZ reporter under control of the ubx promoter, which proves the functional importance of this interaction. Histone H4 specific acetylation has been shown for TAC1 in vitro reflecting the HAT activity of mammalian CBP (Petruk et al., 2001). By targeting CBP acetyltransferase activity to TREs, TRX may increase local acetylation of nucleosomes and thereby increase accessibility of target genes for the basal transcription machinery. Recent evidence suggests that TRX protein is a histone methyltrasferase (HMT) specific for lysine 4 of histone 3 (H3-K4) (Czermin et al., 2002). Even though TRX and ASH1 exhibit largely overlapping chromosomal distributions and can be co-immunoprecipitated from Drosophila embryo extracts (Rozovskaia et al., 1999), ASH1 is not present in the TAC1 complex purified. The suggested function of ASH1 to target TAC1 to specific TREs is supported by the observation that a loss of ASH1 prevents TRX binding to chromatin, and that ASH1 and dCBP physically interact in vitro (Bantignies et al., 2000). The presence of a SET domain in ASH1 finally led to the identification of ASH1 as a HMT specific for K4 and K9 of histone H3 and K20 of histone H4 (Beisel et al., 2002).

The homologue of ASH2 in yeast is a member of the SET1 complex, which has been shown to have H3K4 specific HMT activity (Roguev et al., 2001). This observation led to the proposition that trxG III members therfore are unified by histone H3 K4 methyltransferase activity.

Even though it is still conceivable that further identification of trxG member function include identification as transcription factors in the classical sense, all the trx related proteins identified so far share a different common mode of action. They all perform their maintenance functions via chromatin modifications, namely histone H3K4 methylation and histone acetylation, which are associated with active chromatin, and nucleosome remodelling to facilitate binding of sequence specific transcription factors in actively transcribed regions of chromatin.

I-4   Protein domains in TRX

The *Drosophila* TRX protein is composed of a modular structure with various self contained protein domains (figure I-1a). The most prominent domains in TRX are PHD fingers and a SET domain.

I-4.1   The PHD finger domain

The **P**lant **H**omeo **D**omain (PHD) is a zinc-finger-like motif that has a unique Cys_4-His-Cys_3 pattern, spanning approximately 50 residues (Aasland et al., 1995). The domain folds into an interleaved zinc finger which binds two Zn2+ in a similar manner to that of the RING domain. The structure reveals a conserved zinc-binding core, together with two variable loops that are likely candidates for interactions between the various PHD domains and their specific ligands (Pascual et al., 2000). The possible functions of PHD finger domains include DNA binding, protein-protein interactions or binding of histone modifications (Aasland et al., 1995, O'Connel, 2001; Schultz, 2001) but a single unique function has not been yet documented. Apart from TRX, PHD fingers are also found in ASH1, ASH2 and Polycomb like (PCL), a member of the PcG of proteins as well as many other proteins. All these proteins are implicated in modulation of chromatin structure and gene regulation.

I-4.2   The SET domain

The SET domain (named after the three proteins in which the motif was first discovered: **SU**(VAR)3-9, **E**(Z) and **T**RX) is an evolutionary conserved 150-amino acid sequence motif present in chromosomal proteins from yeast to mammals. First identified in the trxG and PcG gene family, SET domains are also involved in PEV, telomeric and centromeric gene silencing and possibly in determining chromosome architecture (for review see Jenuwein, 2001).

Recently SET domains were found to convey histone lysine MT activity. Proteins like human SUV39H, G9A, SET7, ESET and SET7/9, yeast CLR4, SET1 and SET2 and *Drosophila* E(Z), SU(VAR)3-9 and ASH1 show histone methylation on lysine residues 4, 9, 27 and 36 in histone H3, and on position 20 in histone H4. Most HMTs display site selectivity towards one of the mentioned K residues while others perform dual activity to

(a)

PHD finger   ePHD   ATA1   TAD   ATA2   SET

(b)

AT hooks   SNL1   SNL2   MTase similarity   PHD finger   ePHD   ATA1   TAD   ATA2   SET
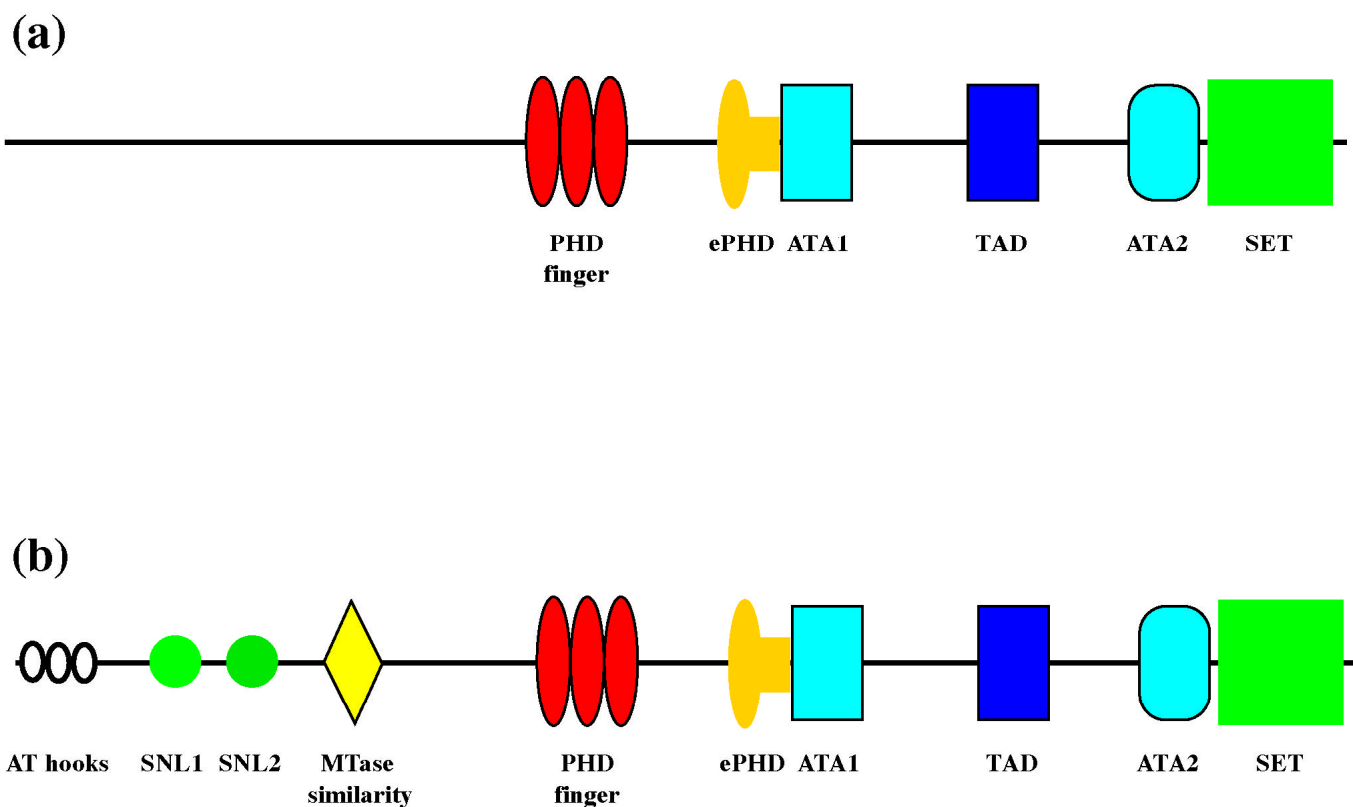
Figure I-1: **Protein domains in** *Drosophila* **TRX and mammalian TRX2.**
(a) TRX protein in Drosophila melanogaster. **(b)** TRX2 protein in mammals (For detailed description please refer to text).
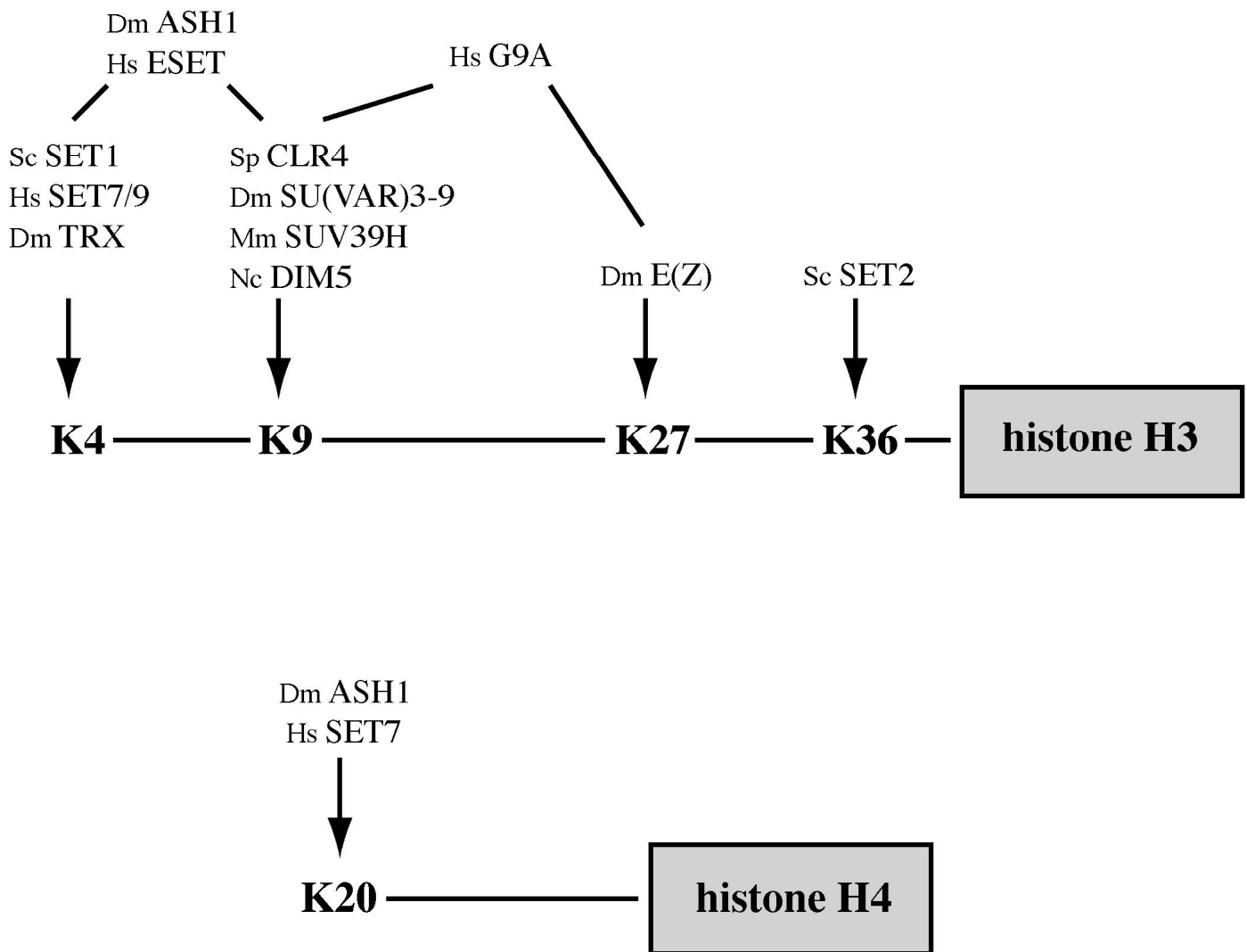
Figure I-2:   Histone lysine methylation.
Overview of selective site-specificity of currently known HMTases for distinct lysine positions in the histone H3 and H4 amino termini (tails). (Modified from Jenuwein and Lachner, 2002)

two different sites (for review see Lachner and Jenuwein, 2002) (see also figure I-2). While regional H3K9 methylation has been shown to be a feature of transcriptionally silent euchromatin (Nielson et al., 2001; Vandel et al., 2001) and constitutive (Jenuwein and Allis, 2001) and facultative heterochromatin (Mermoud et al., 2002; Peters et al., 2001; Boggs et al., 2001), methylation on H3K4 shows a reciprocal relationship, correlating with active chromatin domains (Litt et al., 2001) and in transcriptionally active macronuclei of tetrahymena (Strahl et al., 1999). After several unsuccessful trials with recombinant TRX (Rea et al., 2000) a very recent publication suggests that also *Drosophila* TRX is associated with H3K4 methyltransferase activity.

Recent publication of the crystal structure of SET7/9 (Wilson et al., 2002) and sequence comparison of the SET N-terminal adjacent regions (Roguev et al., 2001) revealed an interesting role for the preSET region. By forming a hydrophobic groove the N-terminal region provides the binding site for the basic side chains of the histone tail substrate and exactly positions the specific lysine residue into the catalytic center of the SET domain. The preSET region in TRX is called ATA2 and there are at least four other types of preSET regions (Roguev et al., 2001).

Besides this HMT activity as a general characteristic of all SET domains, there are also additional functions for TRX-SET reported. They include self-association (Rozowskaia et al., 2000) that might operate in linking TRX proteins residing simultaneously on different maintenance elements, so as to integrate the activity of shared target genes. Also, histone H3 tail binding which is negatively influenced by repressive, but positively influenced by activating, chromatin marks is attributed to the SET domain (Katsani et al., 2001). TRX-SET has also been shown to interact with the *Drosophila* homologue of yeast SNF5, SNR1, a component of the SWI/SNF chromatin remodeling complex (Rozenblat-Rozen et al., 1998), and with ASH1 (Rozovskaia et al., 1999, Roguev et al., 2001).

I.4.3 The TAD domain

The TRX transactivation domain (TAD) is located in the C-terminal part of the protein between ATA1 (to which no specific function is assigned) and ATA2. This domain mediates binding of TRX to dCBP (Ernst et al., 2001) in the 1 MDa histone acetyl transferase (HAT) complex TAC1 that also includes the anti phosphatase Sbf5 (Petruk et

al., 2001). TAC1 acetylates histone H4 tail specifically and is required for correct Ubx expression in vivo (Petruk et al., 2001). ASH1 has also been reported to bind CBP however through N-terminal sequences and the SET domain (Bantignies et al., 2000).

I-5   Opposing actions of PcG and trxG complexes

Both trx-G and Pc-G proteins act at the level of higher-order chromatin structures. Pc-G proteins keep target genes stably and heritably inactive by forming heterochromatin structures. PcG mediated repression involves histone deacetylation (ESC/E(Z)) (Tie et al., 2001; van der Vlag and Otte, 1999), histone methylation by *Drosophila* E(Z) and inhibition of chromatin remodeling by occluding access to nucleosomes for the SWI/SNF complex (PRC1) as a non-catalytic mechanism (Francis et al., 2001, Shao et al., 1999). Important clarity is emerging for trxG/PcG action in the identification of the PcG member **E**nhancer of **Z**este (E(Z)) as a H3 K27 methyltransferase(Mueller et al., 2002; Czermin et al., 2002). Methylation of K27 provides a binding site for polycomb, a chromodomain subunit of PRC1 (Cao et al., 2002), thereby linking histone methylation to Pc-G mediated silencing. Methylation of Lysine 27 correlates with target gene repression (Cao et al. 2002) while H3 K4 methylation mediated by TRX2 leads to chromatin activation. Occurance of similar protein modules in PcG and trxG suggests that both negative and positive chromatin mediated transcriptional regulation of the homeotic genes by these factors involve common principles. Considering the details known about TRX and other TrxG protein members summarized in this introduction, the TrxG might directly antagonize the repressive PcG effects on chromatin structure by applying activating processes like chromatin remodeling (BRM complex), histone methylation on H3-K4 (trxG III) or histone acetylation (TAC1 complex).

The current model for opposing activities of trxG and PcG complexes is summarized in figure I-3 (modified after Simon and Tamkun, 2002), which integrates their nucleosome-modifying roles into the histone code framework introduced by Jennuwein and Allis in 2001. The trxG pathway that leads to gene activation commences with histone acetylation performed by the TAC1 complex (Petruk et al., 2001). Increased acetylation recruits binding of the BRM remodelling complex. (Facilitated binding of SWI/SNF to acetylates nucleosomes has been reported by Hassan et al., 2001). Binding of BRM may be

Me Me   (remodelling ?)   Gene
                                                                              ON
              Me

TRX2
(K4-HMT)   TAC1   Ac   Ac   Ac   BRM   Ac   Ac
              complex   complex
              (HAT)   (remodeling)   Gene
         Ac   ON
                           Ac   Ac   Ac   Ac   Ac

    Ac

              ESC-E(Z)   PRC1
              complex
              (HDAC)   Me
              E(Z)   Gene
              (K27-HMT)   OFF
                           Me   Me

                                               Current Opinion in Genetics & Development
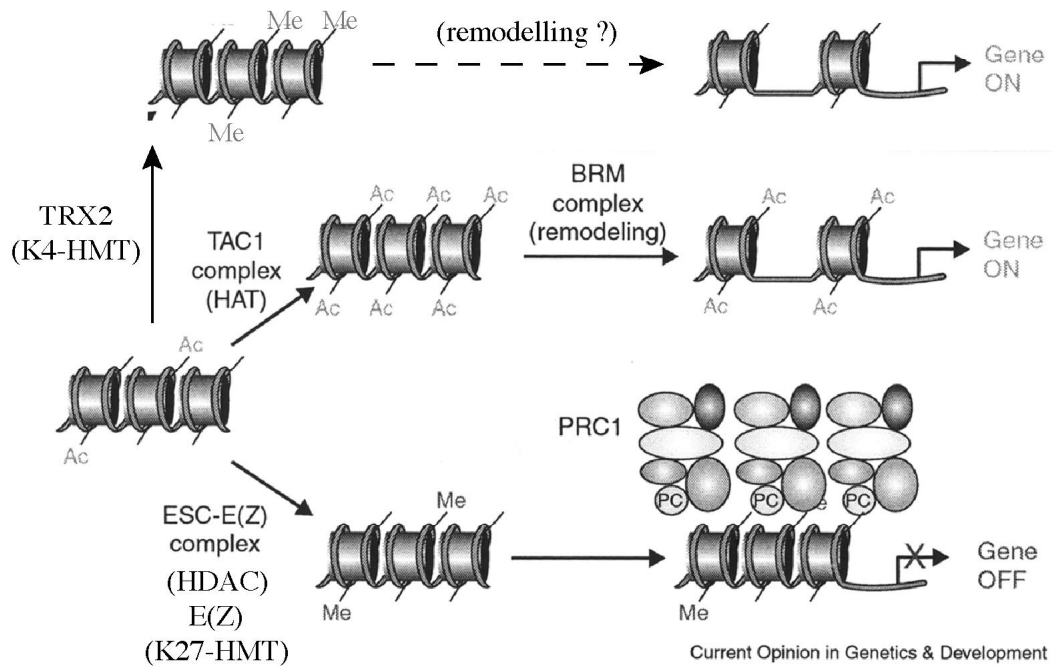
Figure I-3:   Model for multistep mechanism of trxG and PcG complexes (modified Simon and Tamkun 2002).
The cartoon depicts a nucleosomal array at a target gene unter trxG /PcG control. The two trxG activating pathways are shown at the top and the alternative PcG repression pathway is shown below. Ac = acetylation on histone tails. Me = methylation on histone tails.Distinct histone codes created in the first step then help to attract either nucleosome remodelling complexes (trxG pathway) or PRC1 (PcG pathway), whose actions produce the indicated gene expression outcomes. Verticle purple bars indicate opposing effects of complexes. For detailed discussion please refer to text.

mediated by its bromodomain, a module that has been shown to bind acetylated histones (Dhalluin et al., 1999; Zheng and Zhou, 2002). Such initiated nucleosome remodelling renders the DNA more accessible to transcription factors needed for activation (Peterson and Workman, 2000; Vignali et al, 2000). An alternative pathway of trxG activation in also indicated in figure I-3: K4 specific HMT leads to a different histone code which is recognized by the first identified K4 methyl binding protein, ISWI (Kouzarides, unpublished results). This binding has not been shown for the mammalian counterpart BRM, but it is suggestive to think that recruitment of nucleosome remodelling complexes is also involved in this alternative activating pathway.

The first step in the PcG pathway that leads to silencing gene expression features deacetylation of histones by recruitment through the ESC/E(Z) complex (v.d. Vlag and Otte, 1999; Tie et al, 2001) and methylation of histone H3 K27 by E(Z) (Mueller et al., 2002; Czermin et al., 2002). Methylated K27 facilitates binding of PRC1 (Rastelli et al., 1993; Cao et al, 2002). This recruitment potentially includes recognition of the K27 methylgroup by the PC chromodomain (Messmer et al., 1992). Formation of those repressive PRC1 complexes prevents access of remodelling complexes (Shao et al., 1999; Francis et al., 2001) and therefore keeps the DNA region in a closed heterochromatin state.

MAMMALIAN TRX-G

All the trxG members of *Drosophila* have at least one mammalian counterpart. The mammalian genome has two homologs of the *Drosophila* trxG gene brahma and also multiple homologs and anlogs of trx-l and ash1 (Nakamura et al., 2000; Huntsman et al., 1999). TRX has two mammalian homologues named MLL (also called ALL-1, HRX, HTRX) and TRX2 (also called MLL2, MLL4). The similarity in their protein sequence and exon-intron structure clearly indicates that they are paralogous genes (FitzGerald and Diaz, 1999). It appears that the gene duplication event that lead to the existence of two homologous gene copies for trx had occurred in evolution after the divergence of invertebrates and vertebrates. Whether this duplication was linked to the multiplication of the homeotic complexes of which four paralogous groups exist in most vertebrates, remains a topic of discussion.

Comparison to the *Drosophila* TRX protein has revealed multiple conserved regions including the PHD fingers and the C-terminal SET domain. Additional to those sequences there are several protein domains that are unique to mammalian TRX2 and MLL (figure I-1b). Both MLL and TRX2 have three AT-hooks, domains that define sub nuclear protein localization and a domain shared with DNA methyltransferase 1 (DNMT1) in the N-terminal half.

I-6   Protein domains unique to mammalian TRXG

I-6.1   The AT-hook domain

The AT hook is an 11-residue conserved sequence motif capable of binding to the minor groove of AT-rich B-form DNA and was first identified in **H**igh **M**obility **G**roup (HMG) I chromosomal proteins (Huth et al., 1997; Reeves and Nissen, 1990). An Asx bend and cationic R/K bristles that project laterally from the planar backbone of the peptide contribute to the strength of DNA binding. DNA binding of the AT-hook motif is rather based upon structural than upon sequence specific properties.

I-6.2   The NTS domains

Expression of small protein segments of Mll in cultured COS cells defined two **N**uclear **T**ransfer **S**ignals (NTS1 and NTS3) and two sequence stretches that conferred a speckled nuclear distribution (SNL1 and SNL2); (Yano et al., 1997). This nuclear speckled pattern was reasoned to be association of MLL with components of the nuclear matrix, suggesting a role in modulation of chromatin structure, leading to epigenetic effects on the maintenance of gene expression (Caslini et al., 2000).

I-6.3   The MT domain

The presence of a 100 amino acid domain shared with DNA methyltransferase 1 (MT-domain) in mammalian MLL was first discovered by Ma et al. in 1993. Apart from DNMT1 (Yen et al., 1992), this motif is also found the transcriptional repressor MeCP1 (Cross et al., 1997), methyl-CpG binding protein MBD1 (Fujita et al 1999 and 2000) and CpG binding protein hCGBP (Voo et al., 2000). It consists of a cysteine-rich core flanked by patches of basic amino acids. The cysteine-rich part is characterized by the presence of two copies of the signature amino acid sequence CGxCxxC (therefore it is also named CxxC domain). It allows discrimination of methylated, hemi- and unmethylated DNA (Birke et al., 2002).

In the context of MLL, it is part of a larger section that has been dubbed the 'repressor domain' because it can repress transcription if it is artificially recruited to promoters in a fusion with the GAL4 DNA-binding domain (Zeleznik-Lee et al., 1994; Birke et al., 2002). Also GAL4 fusions to the MT domain of DNMT1 have been reported to have a repressive function (Fuks et al., 2000). At least in the case of DNMT1, this repression seems to be connected with histone deacetylation firstly since repression in GAL4 fusions are relieved by trichostatin A (TSA), a known HDAC inhibitor, but also since the two identified DNMT1 complexes contain histone deacetylase I and II respectively (Robertson et al., 2000; Rountree et al., 2000)

The presence of this MT domain involved in either methylating DNA or recognition of methylated DNA points to a connection between TRX2/MLL function and the methylation status of the genome. With help of the MT domain, TRX2 and MLL might be able to sense boundaries of active and inactive chromatin marked by DNA

methylation and, in analogy to DNMT1, recruit protein complexes that would ensure transmission of those epigenetic states over cell generations.

All mentioned domains are self-contained, which might indicate that TRX2 behaves as a modular integrator of different functions relating to chromatin modulation. The combination of AT hooks, SNL, NTS and MT domains suggests mammalian TRX targeting to a specific subset of response elements that contain AT-rich DNA and unmethylated CpGs, an attribute of active promoters. Thereby MLL and TRX could fulfill their function in maintaining pre-activated promoters in an active transcriptional state by applying their abilities as chromatin maintenance factors as mentioned for the *Drosophila* homolog.

I-7   MLL and its involvement in human cancers

Mammalian MLL (**M**ixed **L**ineage **L**eukemia) is a 400 kDa protein ubiquitously expressed during embryonic development. The human Mll gene is located to chromosome 11 in the chromosomal region 11q23 and is involved in a variety of chromosomal translocations in infantile myeloid and lymphoid leukemias (Rowley, 1993; Liang et al., 1996; Look, 1997; Gilliland, 1998). Due to the association with blood cancers it was speculated that wt MLL plays an important role in the early development of the hematopoietic system (reviewed in Ernst et al., 2002). The translocation BCR (break point cluster region) in the Mll gene is tightly confined around intron 11. In the translocations, the N-terminal part of MLL is fused to a broad diversity of fusion partners (reviewed in DiMartino and Cleary, 1999). Since there are no common features of these fusion partners, their role in the tumorgeneric effect of the translocations remains obscure. It seems that two constituents combine to Mll-mediated tumorigenesis. Firstly, removal of C-terminal MLL sequences may create a dominant-negative form (Prasad et al, 1994; Schichmann et al., 1994, 1995) and secondly addition of a fusion partner may provide a positive contribution. The leukemogenic fusion proteins retain the AThooks, and MT domain of the MLL protein while PHD and SET domain are excluded from the fusion. The fusion partner may play roles in stabilization of the fusion protein and/or offering a platform for MLL dimerization (Dobson et al., 2000).

I-7.1 MLL mutant mice

The hypothesis that mammalian MLL has a regulatory function similar to *Drosophila* trithorax has been supported by the phenotypic analysis of targeted disruptions of the mouse Mll locus. To date three different k.o. strategies for Mll have been published :

In the first publication by Yu et al. in 1995, ablated MLL function was achieved by introducing lacZ-polyA into exon three of Mll. This knock out design resulted in truncation of the Mll-transcript behind the three AT hooks. Heterozygous animals were viable but showed a complex phenotype, implying that the Mll gene is haploinsufficient. Heterozygous pups were growth retarded, and displayed female hypofertility, anemia and B-cell population reductions. Segmental abnormalities occured with incomplete penetrance and included sternal malformations and bidirectional axial homeotic transformations. Anterior transformations of cervical vertebra C7 to C6 and thoracic vertebra T3 to T2 as well as posterior transformations of T13 to lumbar vertebra L1 and L6 to sacral segment S1 were reported. At the molecular level posterior shifts of hox-c9 and hox-a7 expression boundaries in E10.5 +/- embryos were detected by RNA in situ analysis. Bidirectional transformations as well as caudally shifted homeotic gene expression boundaries recapitulate the homeotic phenotypes of trx identified in flies.

Homozygous animals were embryonic lethal after E10.5. While spatiotemporal initiation of hox-a7 and hox-c9 expression around E8.0 was still properly established, expression of those genes after E9.0 was completely missing (Yu et al., 1998). This failure of expression maintenance is also analogous to observations for fly trx.

The second mutant Mll allele (Yagi et al., 1998) was truncated after exon 11, which in +/- animals results in only a minor anemia, but otherwise wt appearance. -/- embryos died between E11.5-E14.5 probably due to edema and purpurae which reflects the hematopoietic involvement of MLL in embryonic development. It was proposed that reduced hox expression led to retardation of hematopoietic precursor differentiation and therefore the hematopoietic phenotype. The delayed time of death compared to the first Mll allele could be explained by the differential targeting design: Replacement of exons 12-14 (recapitulating the BCR in human leukemias) with a neo selection marker leads to the expression of an N-terminal transcript that includes AT hooks and the methyltransferase domain and could therefore retain some vital functions of MLL.

Suggestively though this provides a hypomorphic allele that induces delayed but lethal embryonic development.

Ayton et al. published the third Mll-allele in 2001. Their mutant MLL transcript is interrupted after exon five by introduction of STOP-IRES-lacZ. In the heterozygous state, this mutation causes mild skeletal defects but when homozygous embryos never make it beyond the 2-cell stage. Discussions about this difference in phenotypic severity compared to the first two approaches still remain but is explainable by the idea that the third Mll allele creates a dominant negative mutation. In contrast to the first allele, Ayton produces an Mll transcript that contains AT hooks and MT domain. Both domains are known to bind DNA not in a sequence specific but in a structural manner so that the creation of a new dominant negative repressor is conceivable. On the other hand the third allele is the only one that does not create a fusion between the truncated Mll gene and lacZ or neomycin respectively which would lead to a stabilisation of the truncated protein comparable to the situation in chromosomal translocation of human leukemias. Also our unpublished observations with a fourth allele shows, that retrieval of Mll -/- embryos is particulary difficult, indicating that the Ayton allele represents the real Mll knock out. It should be noted though that in neither of the three publications, detection of a truncated protein was successfully investigated.

As targets for MLL the hox genes of cluster hox-a and hox-c could be identified: Yu et al. found that hoxa7 and c9 expression in whole embryos was affected by loss of Mll. Yagi et al. detected shifted expression boundaries for hox-a7, c8 and c9 in unfractionated fetal liver of Mll-/- embryos. Hanson et al. assessed in 1999 the expression of Hox genes in E10.5 **M**ouse **E**mbryonic **F**ibloblasts (MEFs) and found that hox-c4 trough c9 and hox-a3 through a10 were either absent or markedly reduced in Mll-/- MEFs. Interestingly in neither of the investigations, members of the other two hox clusters hox-b and hox-d seem to be altered.

I-8   TRX2

The Mll sister gene Trx2 is located on chromosome 19 (19q13.1) and spans a genomic region of 35 kb (Huntsman et al., 1999; FitzGerald and Diaz, 1999). The 8.5 kb mRNA consists of 37 exons, displays the same exon-intron structure as Mll and expresses to a 280 kDa (2715 amino acids) protein. As heterozygous mutants for MLL show a documented phenotype (see below), TRX2 cannot compensate for the MLL mutation, indicating that their functions are at least partially non-overlapping.

I-7.1 Involvement of TRX2 in human cancers

The Trx2 gene maps to 19q13.1, a region of frequent rearrangements or amplification in solid human tumors (Mitelman et al., 1997). The significant similarity between MLL and TRX2 including the conserved exon-intron structure, sharing of common domains and the location of TRX2 to a site of rearrangements, suggested the potential involvement of this gene in tumorigenesis. Indeed amplifications of the Trx2 gene were detected in two pancreatic cell lines and one glioblastoma cell line (Huntsman et al., 1999). This observation is in concordance with former experiments in *Drosophila*, which showed that dosage regulation of trx is important for hox expression control (Gould, 1997).

I-7.2   TRX2 mutant mice

A Trx2 k.o. mouse is currently being analyzed by Stefan Glaser in the Stewart lab. The mutant Trx2 allele is truncated after exon one. (For detailed description of the k.o. strategy please refer to the results section). Trx2 heterozygous animals are viable, fertile and display no observable phenotype. In the homozygous state the mutation causes embryonic lethality at embryonic stage E10.5, and is characterized by a general retarded growth that is first observed at E7.5 (figure I-4). Gastrulation as a crucial process during embryonic development, in which the three germ layers (ecto-, endo- and mesoderm) of the mammalian embryo are being formed, happens between E6.5 and E7.0. In the subsequent process of neurulation (E7.5 - E8.0) differentiation within the embryonic ectoderm forms the neural plate that will finally lead to development of brain structures and the neural tube. Embryonic stage E7.5 is also characterized by development of extra embryonic structures, which mainly perform important supporting roles for the

developing embryo. In organogenesis that starts around E8.5 development of each of the major organ systems is initiated. The dating of the Trx2 -/- phenotype to E7.5 suggests that knock-out embryos seem to pass unaffected through the gastrulation stages with respect to growth rates. But either aborted pattern formation during gastrulation or failure in important morphological changes (i.e. embryo turning) that take place in subsequent stages of neurulation or organogenesis leads to the retarded growth and finally resorbtion of the mutant embryo. TRX2 seems to have a pleiotropic effect on the essential developmental activities of those stages.

Unverified and unpublished RNA in-situ hybridization studies in our lab have identified the main head organizer gene otx2 and a member of the mammalian Hox gene complexes, Hoxb-1, as potential Trx2 target genes. This data indicates that TRX2 like its *Drosophila* counterpart TRX and its mammalian ortholog MLL is involved in homeotic and Hox gene complex regulation. Interestingly a member neither of the Hox-b nor of the Hox-d cluster has ever been identified as targets for the MLL protein. This might lead to the assumption that TRX2 and its close relative MLL in mammals cooperate to regulate the four Hox clusters; with MLL regulating Hox-a and Hox-c, while TRX2 is regulating Hox-b and Hox-d. Expression of both potential target genes is correctly initiated but not maintained at later stages (our unpublished results). This indicates a further concordance with observations for *Drosophila* TRX, which also acts as a positive maintenance factor.

In summary, the evidence so far indicates that mammalian trxG proteins like the fly counterpart play key roles in the maintenance of expression patterns that are crucial for embryonic development. The way, in which this group of proteins performs that activity, probably involves the maintenance of open or repressed chromatin states.
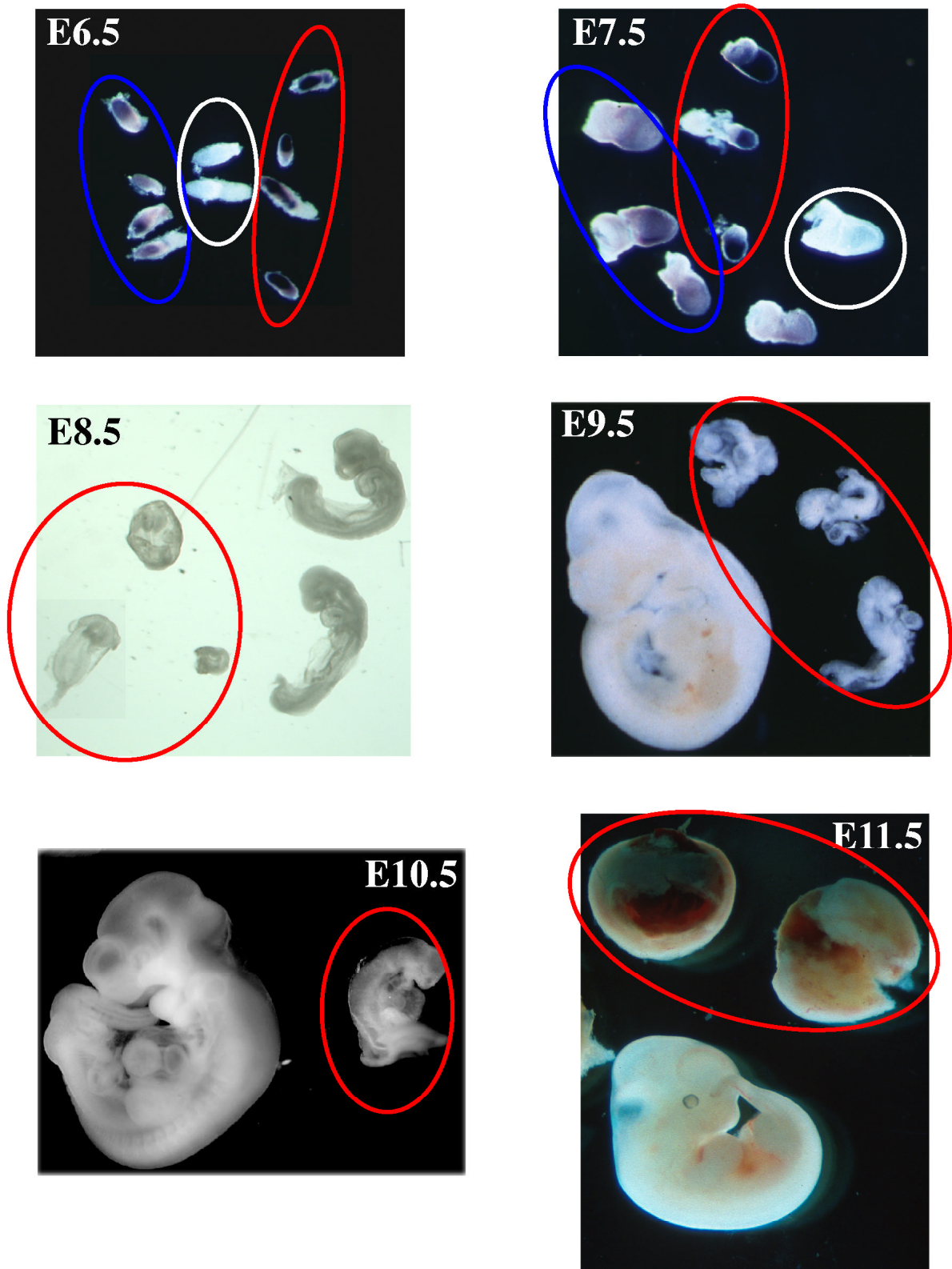
Figure I-4:   Phenotype of Trx2 knock-out embryos.

For developmental stage E6.5 and E7.5 whole mount embryos lacZ stainings are shown. Due to the features of the k.o. cassette the Trx2 k.o. allele can be monitored by lacZ expression. Wt embryos are white (indicated by white oval) while heterozygous (+/-, indicated by a blue oval) and homozygous (-/- indicated by a red oval)) embryos can be distinguished by differentially intese blue staining. For the later stages unstained embryos are shown. Wt and +/- embryos both show wt size while -/- embryos are retarded. Before E7.5 Trx2-/- embryos don't show an abnormal phenotype. From E7.5 on -/- embryos are retarded in size and developmental stage with varying penetrance compared to their wt and +/- littermates. After E10.5 no intact -/- embryo is found. The heavyly retarded red cicled -/- embryos of E11.5 are in the process of resorbtion.

I-8   Goals of this study

The power of genetics in *Drosophila* uncovered an unusual class of transcriptional regulators termed the trxG. Little is known about how trxG members act in mammals. This thesis focuses on one of the mammalian orthologues of the founding member of the trxG, Trithorax. Efforts in our lab to understand the properties of TRX2 in mammals have been confined to early functions in embryonic development since the early lethality of the Trx2 k.o. mouse precludes the study of later functions. Trx2 appears to be expressed ubiquitously throughout development and in the adult. Since the *Drosophila* studies on Trx imply that it plays a major role as a maintenance factor for gene expression patterns, possibly through epigenetic regulation, experimental approaches to examine TRX2 action after the first embryonic crisis caused by its absence were a clear priority.

We chose to use two different ways to explore the involvement of Trx2 in differentiation and fetal development. A Trx2 homozygous knock out ES cell line was created to observe if the loss of Trx2 causes any defects in cell functions and viability in the undifferentiated state. Additionally, studies of the in vitro differentiation potentials of Trx2 deficient ES cells should serve as a model to investigate the role of Trx2 in the differentiation of various cell lineages while Trx2-/- blastocyst injections and analysis of chimeric embryos presented a way to study the role of Trx2 during embryonic development and later stages. Since indications from the fly protein suggest an important involvement in embryonic development on the basis of chromatin regulation through histone methylation, the posttranslational histone modification patterns of Trx2 -/- ES cells were examined.

In a second approach, a fusion of Trx2 with a fluorescent marker by knock-in to the endogenous locus in ES cells was undertaken to examine the cell biology of Trx2 by following the sub cellular distribution of the protein before and during various differentiation stages.

Mat.   Material

Mat-1   Instrumentation

30ml-centrifuge-tubes	Corex
37°C room shaker	Kuehner
37°C shaker (*Multitron*)	Infors
8-channel pipet	Eppendorf
Agarose gel boxes	EMBL workshop
Balance (BP4100S)	Sartorius
Balance (*Explorer*)	Ohaus
Cell culture centrifuge (*Multifuge* 3 S-R)	Heraeus
Cell culture hood (*Herasafe*)	Heraeus
Cell culture incubator (*Heracell*)	Heraeus
Cell culture microscope (CK40)	Olympus
Cell culture rotor (# 6445)	Heraeus
Centrifuge (*Avanti* J-30I)	Beckman/Coulter
Centrifuge 4°C (*Allegra* 6)	Beckman/Coulter
Centrifuge 4°C rotor (GH3.8)	Beckman/Coulter
Centrifuge rotor (JLA-16.250)	Beckman/Coulter
Centrifuge rotor (JA-30.50)	Beckman/Coulter
Centrifuge Table-Top Mini (5415D)	Eppendorf
Digital camera (*CAMEDIA* C-3040Zoom)	Olympus
Dissection fine scisors	F.S.T.
Dissection foreceps (#5)	neoLab
Electroporator bacteria cells (# 2510)	Eppendorf
Electroporator ES cells (*Gene Pulser Xcell)*	BioRad
Fluorescence microscope (BX61)	Olympus
Fluorescence microscope camera (*CoolSnap*)	Visitron Systems
Geiger counter(LB122)	Berthold
Gel documentation system	Biostep
Glass ware	Schott
Hemocytometer	Neubauer
Hybridisation glass tubes	Sauer
Hybridisation oven	Sauer
Ice mashine	Ziegra
Intensifying screens	Sigma
Liquid nitrogen system	Cryo Anlagenbau GmbH
Magnetic stirrer (MR3001)	Heidolph
Microwave	Bosch
PCR mashine (*robocycler gradient* 96)	Stratagene
Phosphoimager (FLA 3000)	Fujifilm
Phosphoimager screens (# 2340)	Fujifilm

Pipetaid	Drummond
Pipettes	Gilson
Platform horizontal shaker	GFL
Platform rocking	EMBL workshop
Power supply	EMBL workshop
Protein gel boxes	Bio-Rad
Protein gel glass plates	Bio-Rad
Rotating wheel	Kisker-Labinco
Scintillation counter	Beckman/Coulter
Semi-dry transfer cell blotter	Bio-Rad
Spectrophotometer (*Ultrospec* 2100pro)	Pharmacia
Spectrophotometer cuvettes	Sigma
Speed vac (*concentrator* 5301)	Eppendorf
Stereomicroscope (MZ8)	Leica
Stereomicroscope (SMZ1500)	Nikon
Stereomicroscope digital net camera (DN100)	Nikon
Thermomixer	Eppendorf
Transilluminator (*BioView*)	Biostep
Ultracentrifuge (*OptiMAX*)	Beckman/Coulter
Ultracentrifuge rotor (MLA80)	Beckman/Coulter
Vacuum oven (*Vacutherm*)	Heraeus
Vacuum system	Vacuubrand
Waterbath	GFL
X-Ray cassettes	Dr. Goos
X-Ray films (*BioMax*MR)	Sigma

Mat-2   Disposables

Aluminium foil	Toppits
Bacteria plates	Greiner
Cell culture freezing vials	Nunc
Cell culture pipetts (5-, 10-, 25-, 50 ml)	Falcon
Cell scraper (23cm)	Nunc
Centrifuge tubes (1.5 ml and 2 ml)	Eppendorf
Centrifuge tubes (12-, 15-, 50 ml)	Falcon
Chromatography paper	Whatman
Combitips	Eppendorf
Cover slips (24 x 60 mm)	Marienfeld
Cover slips (12 mm)	Menzel-Glaeser
Electroporation cuvettes bacteria 1 mm	Eppendorf
Electroporation cuvettes ES cells 0.4 cm	Bio-Rad
Filters (0.22 µm)	Millipore
Filters (*Steritop* 0.22 µm 500ml)	Millipore
Glass beads (1 mm)	Biospec
Gloves	Saenger

Inoculation needles	Nunc
Needles (*Microlance*)	Becton/Dickinson
Nitrocellulose membrane for DNA/RNA transfer (*Biodyne*B)	Pall
Nitrocellulose membrane for protein transfer (*Protran*)	Schleicher + Schuell
PCR tubes (0.2 ml thin walled flat cap)	Peqlab
Saran wrap	Toppits
Screw-cap tubes	Sarstedt
Slides (76 x 26 mm)	Menzel-Glaeser
Spectrophotometer plastic cuvettes	Sarstedt
Syringes	Becton/Dickinson
Tissue culture plates (24-well and 6-well)	Greiner
Tissue culture plates (5-, 10-, 15 cm, 48-well)	Falcon
Tissue embedding molds (22 mm square)	Polysciences Inc.
TLC membrane (*Polygram* CEL 300 PEI)	Macherey-Nagel
Ultracentrifuge tubes (polycarbonate thick wall, 16x64 mm)	Beckman

Mat-3   Chemicals

Acrylamide	Sigma
Agarose	Gibco
Ampicillin	Sigma
APS	Sigma
Bacto-Agar	Difco
Beta-mercaptoethylamine	Sigma
Bisacrylamide	Sigma
Bromphenolblue	Merck
BSA	Sigma
$CaCl_2$	Merck
Chloramphenicol	Sigma
Chromiumpotassiumsulfate	Sigma
Coomassie	Merck
Cyclohexemide	Sigma
DTT	Biomol
EGTA	Sigma
Ethidiumbromide	Sigma
Ferricyanide	Sigma
Ferrocyanide	Sigma
Ficoll	Sigma
G418	Gibco
Gelatine	Merck
Glycine	Merck
HCl	Merck
Hepes	Biomol
Hygromycin B	Roche
IPTG	Sigma

KAc	Merck
Kanamycin sulfate	Sigma
KCl	Merck
KH_2PO_4	Merck
KH_4Ac	Merck
L-Arabinose	Sigma
L-Cystein	Sigma
Methyl green	Sigma
Methylene blue	Sigma
$MgCl_2$	Sigma
Milkpowder	Hairler
Mitomycin	Sigma
$MnCl_2$	Sigma
MOPS	Sigma
Na_2HPO_4	Merck
Na-Citrate	Merck
NaCl	Merck
NaOAc	Merck
NaOH	Merck
N-lauroylsarcosine	Sigma
OCT (tissuetek)	Sacura
Paraformaldehyde	Sigma
PI	Sigma
$RbCl_2$	Sigma
Retinoic acid	Sigma
SDS	Bio-Rad
Sodiumdeoxycholate	Sigma
Spermidintrihydrochloride	Sigma
Sucrose	Sigma
Tetracycline	Sigma
Titriplex (EDTA)	Merck
Trizma	Merck
Urea	Merck
XCFF	Merck
X-Gal	Biomol
$ZnSO_4$	Sigma

Mat-4   Enzymes markers and nucleotides

1kb ladder	Gibco
DNAse I	Sigma
dNTP mix	Amersham-Pharmacia
Klenow large fragment	NEB
Prestained protein marker, broad range	NEB
Proteinase inhibitor cocktail	Sigma

Proteinase K	Merck
Restriction enzymes	NEB
RNA polymerase T3/T7	NEB
RNAse A	Sigma
rNTP mix	Amersham-Pharmacia
T4 DNA ligase	NEB
Taq and PCR buffer	Roche

Mat-5   Solutions and common buffers

<u>LB (Luria Bertoni media)</u>

1%	bacto tryptone
0.5%	bacto yeast
1%	NaCl

<u>PBS</u>

171mM	NaCl
3.4mM	KCl
10mM	Na_2HPO_4
1.9mM	KH_2PO_4

<u>TE (pH 8.0)</u>

10mM	Tris
1mM	EDTA (pH 8.0)

Acrylamide-Bisacrylamide mix (30%)	Severn Biotech LTD
Beta-mercaptoethanol	Sigma
BioRad protein assay	Bio-Rad
DMEM (Glutamax)	Gibco
DMSO	Sigma
ECL solutions	Amersham-Pharmacia
EN^{3}HANCE	NEN
Eosine	Sigma
Ethanol	Merck
Eukitt	Fluka
FCS	Gibco
Formamid	Sigma
Glacial acetic acid	Merck
Gluteraldehyde	Sigma
Glycerol	Merck
Isopropanol	Merck
L-Glutamine	Gibco
LIF-ESGRO	Chemicon
Methanol	Merck
Non essential amino acids	Biochrom
NP40	Roche
Penicillin/Streptomycin	Gibco

Phenol/Chlorophorm/Isoamylalkohol	Sigma
Sodium Pyruvate	Gibco
TEMED	Sigma
TRI Reagent	Sigma
Triton-X100	Sigma
Trypsin/EDTA	Gibco
Tween 20	Sigma

Mat-6   Radioactive isotopes   Amersham-Pharmacia

dATP (redivue desoxyadenosine 5'[^{32}P] triphosphate triethylammonium salt, 10mCi/ml)
dCTP (redivue deoxycytidine 5'[^{32}P] triphosphate triethylammonium salt, 10mCi/ml)
rUTP (Uridine 5'[^{32}P] triphosphate triethylammonium salt, 20mCi/ml)
SAM (S-Adenosyl [L-methyl-^{3}H] methionine, 1mCi/ml)

Mat-7   Antibodies

Anti-beta Actin (monoclonal)	Sigma
Anti-dimethylH3/K4 (polyclonal)	Abcam
Anti-mouse Ig, horseradish peroxidase, from sheep	Amersham-Pharmacia
Anti-rabbit Ig, horseradish peroxidase, from donkey	Amersham-Pharmacia

Mat-8   Kits

Adsvantage-GC cDNA PCR Kit	Clontech
In situ cell death detection Kit, AP	Roche
Plasmid Maxi preparation Kit	Qiagen
Qiaquick PCR purification Kit	Qiagen
Random primed DNA labeling Kit	Roche
Western Star protein detection Kit (AP conjugate)	Tropix

Mat-9   Cells

BL21	Stratagene
EF14 wt ES cell line (mouse strain 129)	
EF14 2.88 (Trx2 heterozygous knockout)	(Frank v.d. Hoeven)
EMFI	Medicore
DH10B/pSC101/BAD/YZA	Genebridges
TG1	Stratagene
XL1-Blue	Stratagene

Mat-10   **Plasmids**

cf28-1   Trx2 cDNA clone
Fe13   16 kb NotI fragment of genomic Trx2 locus
pcDNA3hygro   modified from Invitrogen
pCRIITopo/CF14   contains 5453-5678 of Trx2 cDNA
pEYFP-N1   Clontech
pGex2TK   Amersham pharmacia
X2.2   2.3 kb XhoI fragment of Fe13
X4   4.1 kb XhoI fragment of Fe13
Fe13lacZneo   (Frank v.d. Hoeven)
pSVKeoX1   Angrand et al., 1999
pMC-Cre   Gu et al., 1993

Mat-11   **Probes**
BE580   600 bp EcoRI/BglI fragment of cf28-1
CF14   1238 bp EcoRI fragment of pCRII Topo/CF14 plasmid
EYFP   511 bp PstI/SalI fragment (position 888-1399) of pEYFP-N1
Hygro   632 bp PstI/ScaI fragment (position 2456-3088) of pcDNA3
X4   650 bp HindIII fragment of X4 plasmid

Mat-12   **Syntyhetic oligos (produced by Biospring)**

Orientation of the oligos is 5' – 3'. Restriction target site sequences are displayed in *italics*, PRC primer (annealing) sequences are displayed in **bold**.

ET hygro up
ATATCATCACGAACAGTAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATG**AAAA
AGCCTGAACTCACCGCGACG**
ET hygro dw
GGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTAT
GATCA**CTATTCCTTTGCCCTCGGACGAGT**

E   TTTTTGGATCC**CAGGCACTACCACCACAGCA**
F   TTTTTGAATTC**TCGGGAAGGGACACTCTCAG**

EYFP up
GATCATCC*GGTACC*CGGCCCTGCGCACGGGCTGCCCCTCCCCCCGCCTCCCCGGCCCCTCTCACG
GTGCCAAGATG**GTGAGCAAGGGCGAGGAGCTGTTC**
EYFP dw   GATCATCC*GTCGAC*C**CTTGTACAGCTCGTCCATGCC**

Trx2 up   GATCATCC*CTCGAGA*GCGGCGGCGGCGGGCGGCGGCAGT
Trx2 dw   GATCATCC*GAGCTC***CTGAACTCGCGCACCAGCCC**

L   GGCCCCTCTCACGGTGCCAAGATG
M   CTCCGGCATGCAGCCTCGGTTCGG

55

M. Methods

WORKING WITH DNA

M-1 Restriction enzyme digestion

Equimolar amounts of DNA fragments are cleaved with a convenient restriction enzyme.
For calculation of the amount of enzyme, the following rule is applied :
1 unit of restriction enzyme cuts 1 µg DNA within 1 hour.
Every enzyme is used with its corresponding buffer in 1x concentration.
The optimal temperature and further information about conditions for every digest is
gathered from the *New England Biolabs* (NEB) catalogue.
Incubation is at least 2 hours and the reaction volume is usually 20 µl.

M-2 Ligation

Ligations were set up by mixing vector and insert DNA (insert in three fold molar excess to
vector) in a volume of 15 µl with one unit DNA T4 ligase and the buffer suggested by the
supplier. The reaction was incubated at 16°C o/n.

M-3 Transformation
M-3.1 Preparation of heat shock competent cell

2x salt :	120m	$MCaCl_2$	buffer:	1x	salt
	80m	M KAc		45mM	$MnCl_2$
	30%	Sucrose		100mM	RbCl
	adjust pH with HCl to 5.6 - 5.9, filter sterile and			in H_2O (prepare fresh each time)	
	keep at -20°C.				

- Grow cells on agar plates containing the appropriate selection o/n.
- Start overnight culture in the appropriate selection o/n.
- Dilute 1/50 in LB and grow to OD_{600} 0.35.
- Cool cells in ice water
- Spin 3000 rpm at 4°C for 10 min (*Allegra* 6 rotor GH3.8).
- Resuspend pellet in 40 ml ice-cold buffer.
- Spin 3000 rpm at 4°C for 10 min.
- Resuspend pellet in 20 ml ice-cold buffer and add 500mM DMSO
- Divide into 200-µl aliquots
- Snap freeze in liquid nitrogen, and store at -80°C

M-3.2 Heat shock transformation
- Thaw competent cells on ice.
- Add about 10ng DNA in 5 µl H_2O.

- Leave 15 min on ice.
- Heat shock 1 min, 40 s at 42°C.
- Leave the tubes for 4 min on ice then add 800µl LB-media
- Leave 30 min at 37°C shaking at 12000 rpm.
- Spin down 4000 rpm for 4 min at RT.
- Take off 850µl, resuspend cell pellet in the remaining 150µl and plate on agarose plates containing the appropriate selection marker.

M-4   Mini preparation of plasmid DNA

resuspension buffer: 50mM   Tris-HCl, pH 8   lysis buffer: 200mM   NaOH
                      10mM   EDTA   1%   SDS
                      100 µg/ml RNAse A

neutralization buffer: 3 M   potassium acetate

- Transfer 1 ml of o/n culture into an Eppendorf tube.
- Spin 16,000 x g at RT for 2 min, remove supernatant and resuspend the bacterial pellet in 400µl resuspension buffer.
- Add 400 µl lysis buffer, mix by inverting.
- Add 400 µl neutralization buffer, mix by inverting.
- Leave 15 min at RT to let the RNAse in P1 work, then cool 5 min on ice.
- Spin at 16,000 x g for 3 min.
- Transfer supernatant, containing plasmid DNA, into a 2- ml Eppendorf tube.
- Precipitate DNA to remove residual salt by adding 900 µl isopropanol, invert
- Spin 16,000 x g at RT for 10 min.
- Wash pellet with 70% ethanol
- Vacuum dry for 5 min in the speed-vac
- Resuspend in 35µl H2O or TE and use 1µl for analytic digests.

M-5   Maxi preparation of plasmid DNA (*QIAGEN*)
The principle of this method is based on alkaline lysis of the bacterial cell, followed by binding of the plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins and low-molecular-weight-impurities are removed by a medium salt wash. Plasmid DNA is eluted by a high–salt buffer, and then concentrated and desalted by isopropanol precipitation.

P1 buffer: 50mM   Tris-HCl, pH 8   P2 buffer: 200mM   NaOH
           10mM   EDTA   1%   SDS
           100 µg/ml RNAse A

P3 buffer: 3 M   potassium acetate   TE: 10mM   Tris-HCl, pH 8
                                                      1mM   EDTA

QBT buffer: 750mM   NaCl
                   50mM   MOPS
                   15%   isopropanol
                   0.15%   triton X-100

QC buffer:	1 M	NaCl		QF buffer:	1.25 M	NaCl
	50mM	MOPS			50mM	Tris, pH 8.5
	15%	isopropanol			15%	isopropanol

- Inoculate a starter culture of 200 ml LB medium, containing the appropriate selective antibiotic, and grow o/n at 37˚C, shaking 300rpm.
- Harvest the bacterial cells by centrifugation 6,000 x g for 10 min at 4˚C.
- Resuspend the pellet in 10 ml buffer P1.
- Add 10 ml buffer P2, mix gently by inverting, then incubate at RT for 5 min
- Add 10 ml buffer P3. Mix immediately by inverting, then incubate on ice for 20 min
- Spin 20,000 x g, for 30 min at 4˚C, and remove supernatant containing plasmid DNA.
- Equilibrate the QIAGEN-tip 500 by applying 10 ml QBT buffer, and allow the column to empty by gravity flow.
- Apply the supernatant to the column and let it enter by gravity flow
- Wash twice with 30 ml QC buffer then elute DNA with 15 ml buffer QF.
- Precipitate DNA by adding 10.5 ml (= 0,7 volumes) isopropanol to the eluted DNA. Mix and spin at 15,000 x g, 4˚C for 30 min.
- Wash pellet with 70% ethanol, and spin at 10,000 x g for 10 min.
- Dry the pellet and redissolve the DNA in a suitable volume of water or TE.

M-6   DNA precipitation

TE:	10mM	Tris-HCl, pH 8
	1mM	EDTA

- Add sodium acetate to a final concentartion of 0.3M, mix by inverting.
- Add to a final concentration of 70% ethanol, mix by inverting.
- Spin 16,000 x g at 4°C for 15 min.
- Decant the supernatant and wash pellet with 70% ethanol.
- Spin 16,000 x g at 4°C for 15 min, and decant supernatant.
- Vacuum-dry pellet and resuspend in an appropriate volume of H_2O or TE.

M-7   Phenol-Chloroform extraction

To remove proteins from your DNA mix, the sample has to be phenol extracted. After adding phenol/chloroform and mixing, proteins will enter the organic phase, while DNA will stay in the aqueous phase.

- Add 1 volume phenol/chloroform/isoamylalkohol (25:24:1) and vortex for 10s.
- Spin 16,000 x g at RT for 5 min.
- Transfer upper water-layer into new tube and ethanol-precipitate.

M-8   Agarose gel electrophoresis

10x DNA loading buffer:	25%	Ficoll		TBE:	0.45 M	Tris	
	100mM	EDTA			0.45 M	boric acid	
	BFB and XCFF				1mM	EDTA	pH8

Molecules of linear or circular DNA become orientated in an electric field and migrate through a gel matrix (in this case agarose) at rates that are inversely proportional to the size of the linear fragment. Larger molecules migrate more slowly because of greater frictional drag and because they find their way through the pores of the gel less efficiently than smaller molecules. Superhelical, nicked circular and linear DNAs of the same molecular weight migrate at different rates. The migration speed of these different forms depends mainly on the agarose concentration in the gel and the concentration of intercalating dye included.

The gels used in this thesis to analyse plasmid-DNA contain 0.8% agarose and 20ng/ml ethidium bromide. For all gel-runs with plasmid DNA, 1xTBE was used as running buffer. DNA samples were mixed with 1x DNA loading buffer. To obtain maximum resolution of the DNA fragments, gels were run at 5 V/cm.

M-9   ET cloning

In *E.coli*, the classical homologous recombination pathway involves RecA as a strand invasion protein and the RecBCD complex as the major cellular exonuclease. Since RecBCD also plays a role in recognition and destruction of foreign linear DNA, it is impossible to introduce a linear fragment of DNA for homololgous DNA engineering. Hence homologous recombination in *E.coli* has proven to be a diffecult process.

The alternative to the recA based pathway is ET/Red recombination developed by Zhang et al. in 1998. Homologous recombination is initiated by either of the two functionally equivalent protein pairs: RecE/RecT from the Rac phage and Redα/Redβ from the λ phage (Zhang et al., 1998; Muyrers et al., 1999). RecE and Redα are 5' 3' exonucleases, while RecT and Redβ are DNA single strand annealing proteins (Muyrers et al., 2000). They serve to circumvent both RecA and RecBCD whereas RecBCD- strains can be used. Alternatively, Rec BCD can be inhibited by expression of the Redγ protein so that use of linearized DNA is possible.

In the fundamental reaction, a linear DNA molecule carrying a selectable marker flaked by 40-60 bp regions of sequence homologous to the desired integration location on a circular DNA molecule. The recombinogenic, linear DNA fragment can be generated by PCR with oligonucleotides containing 3' the PCR primer sequence and 5' the 40-60 bp homology arm.

ET/Red recombination has proven to be successful in cloning of regular plasmids, BACs and the E. coli chromosome with a very high efficiency rate. In this thesis ET cloning was applied to modify the knock out cassette for Trx2 and for the creation of the EYFP-Trx2 targeting cassette.

M-9.1 PCR reaction and recipient plasmid DNA preparation

- PCR mix: 10-20 ng   template DNA
            1 μM   5' primer and 3' primer each
            1x   PCR reaction buffer (Roche)
            0.2mM   dNTP
            2.5 units   Taq polymerase (Roche)
            fill up with dH_2O to 50 μl final volume.

- Perform PCR in Stratagene robocycler:

  PCR protocol: 3 min 94°C 1 min 94°C

  40 sec 62°C

  1 min 72°C   35 cycles

  10 min 72°C

- Pool PCR products, add 400 U DpnI, 1x NEB-buffer4 and fill up with dH_2O to 150 µl volume.
- Perform DpnI digest at 37°C for two hours to remove residual template DNA.
- Ethanol-precipitate digestion-mix and resuspend in 10 µl dH_2O.
- For cotransformation extract recipient plasmid DNA with Phenol/Chlorophorm and resuspend in in dH_2O at 1 µg/µl.

M-9.2 Preparing electro-competent cells
- Prepare 10% glycerol with dH2O, and cool down for at least three hours before usage.
- Grow 5 ml o/n cultures of DH10B-YZA cells in 5 µg/ml tetracycline.
- Make a hole in the lid of an eppendorf tube and dilute o.n culture 1:50 in 1.4 ml fresh selective medium.
- Grow for 2 hrs at 30°C with shaking to an OD_{600} of 0.2.
- Add arabinose to a concentration of 0.1-0.2%.
- Transfer to 37°C and continue growing for about one hour until cells reach log phase (OD_{600}= 0.35-0.4).
- Spin down the cells at 11,200 x g for 30 sec at 4°C.
- Discard the supernatant, place the tube on ice and resuspend pellet in 1 ml icecold 10% glycerol.
- Spin down the cells at 13,400 x g for 30 sec at 4°C.
- Discard the supernatant, place the tube on ice and resuspend pellet in 1 ml icecold 10% glycerol.
- Spin down the cells at 13,400 x g for 30 sec at 4°C.
- Discard the supernatant, leaving 20-30 µl to resuspend pellet in the remaining solution.
- Use cells immediately for best transformation efficiency or snap freeze in liquid N and store at -80°C.

M-9.3 Electro-Transformation
- Precool 1-mm-cuvettes on ice for 5 min.
- Thaw electrocompetent and arabinose-induced cells on ice (or use them straight after preparation). Add 1 µl PCR product (corresponds to 2-3 µg/0.2-0.3 pmol) and 1 µl recipient plasmid (corresponds to 1 µg/0.1-0.2 pmol).
- Co-Electroporate the cells at 1200 V.
- Immediately add 1 ml LB medium and transfer back into eppendorf tube.
- Incubate at 30°C for 70 min shaking at 1200rpm.
- Spin at 5000 rpm for 2 min, aspirate the supernatant, but leave 100 µl liquid.
- Resuspend the cells in the remaining liquid, plate on appropriate antibiotic and incubate o/n at 37°C.

M-10   Total RNA extraction
- Grow ES on 10-cm-dish to confluency.
- Wash once with PBS, then add 1 ml TRI REAGENT™ and collect cell lysate with a sterile cell scraper. Pass several times through a pipette to form a homogenous lysate.
- To ensure dissociation of nucleoprotein complexes, let samples stand for 5 min at RT.
- Add 0.2 ml chlorophorm per ml of TRI REAGENT™ used.
- Shake vigorously for 15 sec and allow to stand for 2-15 min at RT
- Centrifuge at 12,000 x g for 15 min at 4°C. Centrifugation separates the mixture into 3 phases: a organic phase containing protein, an interphase containing DNA, and a colorless upper aqueous phase containing RNA.
- Transfer the aqueous phase to a fresh tube and add 0.5 ml isopropanol per ml of TRI REAGENT™ used and mix.
- Allow the sample to stand for ten min at 4°C.
- Centrifuge at 12,000 x g for 10 min at 4°C. The RNA precipitate will form a pellet on the side and bottom of the tube.
- Remove the supernatant and wash the RNA pellet by adding 1 ml 75% Ethanol per ml of TRI REAGENT™ used.
- Vortex the sample and centrifuge at 12,000 x g for 5 min at 4°C.
- Samples can be stored in ethanol up to one year at -20°C.
- Air-dry the RNA pellet. Dissolve in 50 µl RNAse free H_2O, aliquot and store at -80°C.

M-11   DNA extraction from ES cells

lysis buffer: 50mM   Tris-HCl, pH 8
                 100mM   EDTA
                 100mM   NaCl
                 1%   SDS
                 add fresh just before use 0.5 mg/ml Proteinase K

- Remove media from confluent layer of ES cells in 24-well plate. This amount of cells will yield about 30-40 µg genomic DNA.
- Add 500 µl lysis buffer and incubate o/n at 37°C.
- Take the 500 µl DNA containing cell lysate off the wells and transfer into 1ml-tubes
- Mix 5 min on Eppendorf mixer
- Add 200 µl conc. NaCl (~ 6M) and shake by hand very hard for 2 min
- Spin for 5-10 min 16,000 x g at RT.
- Take 550 µl without top phase and pellet into new tube
- Add 400 µl isopropanol, mix 2 min on Eppendorf mixer and spin for 2 min 16,000 x g.
- Take off supernatant and wash with 70% Ethanol. Spin again for 4 min.
- Take off supernatant and drain tube on piece of tissue. Remove residual ethanol with a pipette and let pellet dry for not longer than 2-5 min. The pellet should still be wet
- Then add 100 µl TE and incubatefor 2 hrs at 37°C with gentle shaking.
- The average DNA concentration should be 0.5-1 µg/µl.
- For test digest use 25 µl DNA and 20 U enzyme in a total volume of 45 µl.
- For genomic PCR use 1µl in 50 µl volume. For genomic Southern use 25 µl in 45 µl volume.

M-12 DNA extraction from mouse-tails (yields about 100 µg genomic DNA)

lysis buffer:

50mM	Tris-HCl, pH8	
100mM	EDTA	
100mM	NaCl	
1%	SDS	
0.5mg.ml	proteinase K (add freshly before use)	

TE:

10mM	Tris-HCl, pH 8
1mM	EDTA

- Add 400 µl lysis buffer to the mouse-tail and incubate at 55˚C o/n
- Add 400 µl phenol/chlorophorm/isoamylalcohol (25:24:1) and incubate in a rotating wheel for one hour.
- Spin 9,300 x g for 10 min at RT.
- Transfer supernatant into new tube, add 400 µl chlorophorm and incubate again for one hour in a rotating wheel.
- Spin 9,300 x g for 10 min at RT.
- Transfer supernatant into new tube and add 3M potassium acetate, pH5.5 to 5%.
- Mix and add isopropanol to 70-80%. Shake hard by hand to precipitate DNA and let the DNA sink to the bottom of the tube.
- Take off supernatant without disturbing the DNA and wash pellet with 70% Ethanol.
- Spin 9,300 x g for 5 min at RT.
- Discard supernatant. Remove excess liquid by pipetting and air-dry for 2-5 min.
- Resuspend DNA pellet in 200 µl TE. Store at 4˚C.
- Use 1 µl for genomic PCR, 25 µl for genomic Southern.

M-13 Polymerase Chain Reaction (PCR)
The PCR reaction is classified into 3 major steps :
1. Denaturation of the DNA template
2. Primer annealing
3. Polymerisation
All steps were performed in consecutive cycles in a *Stratagene* Robocycler PCR mashine.
The PCR product is a double stranded DNA, consisting of the region of the complementary strain between the flanking primers.

M-13.1   Reaction-mix

PCR mix:	10 ng	template DNA
	1µM	upstream (5') and downstream (3') primer
	1x	PCR reaction buffer
	0.2mM	dNTP
	2.5 units	Taq polymerase

M-13.2   Protocol
Reaction conditions vary among different experiments, but are usually conformed to the following cyclic pattern: 1 min initial denaturation at 94˚C, 35 cycles consisting of 1 min denaturation at 94˚C, 1 min annealing at the relevant temperatures (depending on the primer composition) and variable times of extension at 72˚C depending on the size of the PCR product. The last cycle was by 10 minutes of additional extension at 72˚C.

M-13.3   PCR purification (*QIAGEN*)

To extract the PCR product for further working, the PCR Purification Kit protocol from *QIAGEN* was applied.

DNA ranging from 80bp to 10 kb binds to silica-membrane in the presence of high salt concentrations. Short oligos (most primers), nucleotides, salts and polymerases pass through, while the PCR product is eluted with TE or H2O.

- Add 5 volumes buffer PB to one volume of the PCR reaction and mix.
- To bind DNA, apply the sample to the QIAquick column and centrifuge 10,000 x g for 30 sec.
- Discard flow-through. To wash add 750 µl buffer PE and spin 10,000 x g for 30 sec.
- Discard flow-through and spin an additional time at 10,000 x g for 60 sec.
- Place QIAquick into a clean 1.5-ml eppendorff tube, add 50 µl TE, let stand for 5 min and spin 10,000 x g for 30 sec to elute DNA.

M-14 Southern analysis

In the southern analysis DNA from a standard agarose gel is transferred onto a nylon membrane. The membrane can be incubated with a hybridisation probe that binds a specific DNA fragment. In this case the Southern blot was carried out to verify homologous integration of the targeting constructs into the genomic Trx2 locus.

M-14.1   Gel run and southern blotting

50x TAE:	2M	Trisma Base	20x SSC:	3M	NaCl
	10mM	EDTA		0.3M	sodiumcitrat
	0.57%	glacial acetic acid			adjust with NaOH to pH

- Digest about 7-10 µg of genomic DNA prepared from ES cells with the desired restriction enzymes o/n in a reaction volume of 45 µl.
- Add 1x gel running buffer and load onto a 0.6% TAE gel.
- Run gel in 1x TAE o/n at 4°C.
- Wash gel three times for 25 min in 0.4M NaOH.
- Wash gel for 20 min in 20x SSC.
- Blot onto a nitrocellulose membrane in 20x SSC o/n.
- Mark slots on the membrane and vaccuum dry for 3 hours at 80°C.

M-14.2   Radioactive 1kb ladder

- Mix:
	mM	dGTP, dCTP, dTTP Mix	
	2µg	1kb ladder	
	1x	NEB2	
	20 µCi	^{32}P dATP	
	fill up with H$_2$0 to 20 µl		*1
	5 units	DNA Pol I large (Klenow) fragment	
- Incubate for 20 min at 37°C.   *2
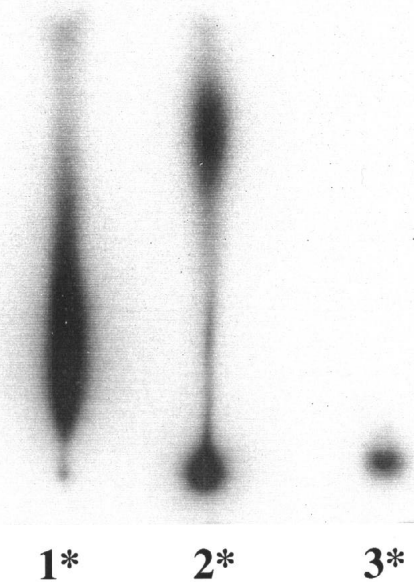- Heat inactivate the Klenow enzyme at 65°C for 10 min.

Figure M-1: Autoradiograph of radioactive 1 kb ladder preparation.
1 kb ladder fragments created by EcoRI digestion contain overhangs that are filled by Klenow enzyme with radioactively labeled dATP nucleotides. Single 0.1 yl samples were spotted and separated by thin layer chromatography (TLC). 1*= Sample before Klenow reaction. Free radioactive nucleotides are not yet incorporated. 2*= Sample after Klenow reaction. Incorporated nucleotides remain on the origin, left over free nucleotides are separated. 3*= Sample after precipitation. Free nucleotides are removed. The sample only contains incorporated radioactive label.
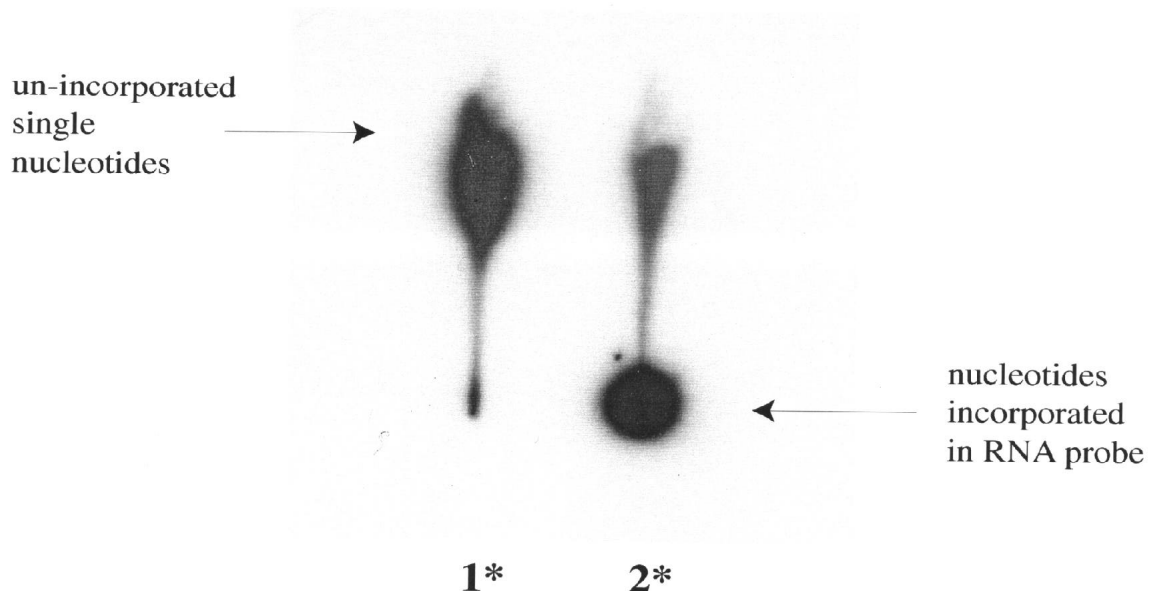

Figure M-2: Autoradiograph of radioactive RNA probe preparation.
T3 polymerase uses radioactively labeled dUTPs to produce a labeled RNA transcript. Single 0.1 yl samples were spotted and separated by thin layer chromatography (TLC). 1*= Sample before polymerase reaction. Free radioactive nucleotides are not yet incorporated. 2*= Sample after polymerase reaction. Incorporated nucleotides remain on the origin, left over free nucleotides are separated.

Ethanol precipitate and dissolve DNA pellet in 100µl TE. Store the ladder at -20°C and use about 5000 cpm per lane in 1x loading buffer.   *3

* = spot 0.1 µl on a filter membrane for TLC, dry, run the TLC with KH_2PO_4 and expose the filter for 5 min. The picture must show the pattern displayed in figure M-1.

M-14.3   Radioactive RNA probe

T3 or T7 polymerase reaction is used to transcribe a desired DNA fragment. By adding radioactive labelled dUTP to the transcription reaction a radioactive RNA-probe is produced.

- Transcription-mix: 1µg   linearized DNA
                            1x   transcription buffer (Stratagene)
                            1mM   rATP, rGTP, rCTP
                            60 µCi UTP^{32}
                            5 units RNAsin   *1
                            5 units T3 Polymerase

- Incubate at 37°C for 15 min.
- Add 5 units RQ-DNAse to destroy the template for transcription.   *2
- Incubate at 37°C for 15 min.

* = spot 0.1 µl on a filter membrane for TLC, dry, run the TLC with KH_2PO_4 and expose the filter for 5 min. The picture must show the pattern displayed in figure M-2.

M-14.4   Radioactive DNA probe (random primed DNA labeling kit (*Roche*)

- Denature 50-100 µg DNA fragment containing the desired probe sequence in a 9 µl volume by heating to 100°C for 10 min, and subsequent cooling on ice.
- Add 25 nM dATP, dGTP and dTTP solution.
- Add 1x reaction buffer (*Roche*).
- Add 50 µCi [^{32}P]dCTP, aqueos solution.
- Add 2units Klenow enzyme (*Roche*).
- Incubate for 60 min at 37°C.
- For precipitation add   50mM EDTA
                             20 µg tRNA
                             4.3M NH_4Ac
                             75%   Ethanol
- Precipitate for 5 min at RT.
- Spin 5 min 16,000 x g at RT.
- Wash pellet with 70% ethanol, dry shortly and dissolve in 100 µl TE.
- For denaturing add 0.5M NaOH and leave for 15 min at RT. Use volume according to about 10^7 counts per 100cm^2 membrane immediately.

M-14.5 Hybridisation

hybridisation buffer: 7% SDS
250mM Na$_2$HPO$_4$ pH 7.2
1% BSA
1mM EDTA

wash buffer: 20mM NaH$_2$PO$_4$ pH 7.2
1% SDS
1mM EDTA

- Wet the membrane from step M-14.1 in 25mM NaHPO4 and roll it into a glass tube.
- Add 10 ml Hybridisation buffer to the tube and pre-hybridise for 30 min in a turning oven at 72°C for RNA probes, at 66°C for DNA probes.
- Dilute the prepared probe (M-14.3 or M-14.4) in 10 ml hybridisation buffer and add to the membrane in the glass tube.
- Place the tube back into a turning oven and hybridize at appropriate temperature o/n.
- Replace the probe solution with wash buffer, rinse and wash for 10 min, then 30 min then 1 hour in the turning oven at appropriate temperature to remove residual unbound probe.
- Expose membrane o/n.

M-15 Northern blotting

10x MOPS: 200mM MOPS
50mM Na-acetate
10mM EDTA
adjust pH 7.0 with NaOH

20x SSC: 3M NaCl
0.3M sodiumcitrat
adjust with NaOH 10N to pH 7

50x Denhardt: 1% Ficoll type 400
1% BSA fraction5
in H$_2$O

LB stock: 10% Ficoll type 400
0.1% bromphenolblue
in H$_2$O

H-Mix: 50% formamid
5x SSC
50mM NaP pH 6.5
8x Denhardt
0.5 mg/ml yeast RNA
1% SDS

RNA sample buffer: 53% formamide
6.7% formaldehyde
1x MOPS
18% L.B. stock

Northern gel: 0.8% agarose
1x MOPS
6% formaldehyde

wash buffer1: 2x SSC
1% SDS

wash buffer2: 0.2X SSC
0.1% SDS

- Use 30μg total RNA (M-10) per lane.
- Calculate volume of RNA solution and add 2.35x volume RNA sample buffer.
- Leave 15 min at 65°C then put on ice.
- Run gel in running buffer (1xMOPS) at 230 V for two hours.
- If desired stain gel in 0.5μg/ml Ethidiumbromide for 30 min, take picture and destain in H$_2$O for 30 min.
- Blot in 10x SSC o/n. Use nitrocellulose membrane BiodyneB.
- Rinse membrane in 2x SSC.
- Air-dry on 3mm paper then vacuum bake for 3 hrs at 80°C.
- Wet filter in 5x SSC and transfer to hybridization tube, add 15 ml prewarmed H-Mix and prehybridize for 1-3 hrs at 42°C in a rotating wheel.

- Remove prehybridisation solution and add 15 ml fresh prewarmed H-Mix containing denatured random primed DNA probe.
- Hybredize o/n at 42°C in a rotating wheel.
- Wash 15 min at RT in wash buffer1, then 2x 20 min at 65°C in wash buffer2.
- Expose to X-Ray film.

WORKING WITH PROTEINS

M-16 Crude protein extracts

buffer E :

20mM	HEPES pH 8.0	
350mM	NaCl	
10%	Glycerol	
0.1%	Tween 20	
1%	Proteinase inhibitor cocktail (Sigma)	add freshly
2mM	EDTA	add freshly

- Remove media from ES cell dish and wash once with PBS.
- Add icecold PBS and collect cells with a sterile scraper. Wash plate once with icecold PBS and add to the collected cells.
- Spin 5 min 1000 rpm at 4°C, remove supernatant and resuspend cell pellet in 1 ml icecold PBS. Transfer to 2ml-Eppendorff tube.
- Spin 2 min 5,900 x g, remove supernatant, shock-freeze cells in liquidN. Store at –80°C.
- Add about 500 µl ml buffer E to frozen cell pellet (leave cells in liqid N until use) resuspend carefully and combine cell suspensions from same cell identity.
- Transfer solution into plastic vial filled to 70% with 1-mm-glass-beads (sterile), close lid without airbubbles
- Bead-beat for 30 sec at 5000 rpm then cool two min on ice. Repeat six times.
- Leave on ice, spin down at 16,000 x g for one min at 4°C.
- Take out 800 µl supernatant transfer to precooled polycarbonate thick wall ultracentrifuge tubes (6.5 ml /16x64 mm/Beckmann /355647) on ice.
- Add 500 µl cold buffer E, mix, spin again and add 500 µl supernatant to the corresponding ultarcentrifuge tube.
- Ultarcetrifuge 100,000 x g for 1 hour at 4°C in a MLA80 rotor.
- Aliquot at 4°C and immediately shock freeze in liquid N. Store at –80°C. (Use approximately 40 µl protein extract from 10 x 15-m-plates for one small western gel)

M-17 Nuclear extracts

Nuclei extracion buffer:	15 mM	Tris-HCl pH 7.5
(100 ml)	60 mM	KCl
	15 mM	NaCl
	5 mM	MgCl$_2$
	0.5 mM	EGTA
	300 mM	Sucrose
	1%	NP40
	add 0.5mM -mercaptoethanol fresh before use	
	add 1% proteinase inhibitor cocktail fresh before use	

- prepare 2 buffers, one containing, the other without NP40.
- Grow ES cell on gelatinized10 cm plate without feeders.
- Trypsinize or collect cells with a sterile cell scraper and spin down cell pellet at 1000 rpm for 5 min (*Multifuge* 3 S-R, rotor 6445).
- Put cells on ice and resuspend in 1 ml extraction buffer without NP40 per 2-10 x 10^6 cells. Add same volume NP40 buffer and mix carefully, leave on ice.
- Leave on ice for 20 min. (Clean nuclei are smooth and refract light. If nuclei are not released from cells try (i) douncing (loose fitting pestle) and/or (ii) pipetting up/down repeatedly through a small bore pipette)
- Spin down nuclei 1500 rpm for 5 min at 4°C.
- Remove supernatant and store nuclei pellet at -80°C.

M-18 Histone labelling

MFM media:	5%	dialysed FCS
	2mM	L-Glutamine
	100 u:µl/ml	Penicillin/Streptomycin
	1000U/ml	LIF
	48µg/ml	L-Cystein
	in DMEM without L-Methionine and L-Cysteine (Gibco)	

- Grow ES cells on feeders. Three days before the planned experiment preplate and split onto gelatine coated 10-cm-dish.
- One day before the labeling experiment, split the cells into fresh complete tissue culture medium. (Set up the split ration to reach 70-80% confluency for labelling. The cultures should contain 10^6 to 10^7 cells.)
- On the day of the experiment, replace the culture medium with the minimum volume MFM (e.g. 3 ml for a 10-cm-dish). Incubate one hour.
- Add 200 µCi [^{3}H-methyl]methionine per ml MFM. Incubate eight hours.
- Remove radioactive media, wash twice with PBS and nuclei extract. (To equalize cell numbers for individual cell clones keep a second plate for every clone that you treat the same way, except leave out radioactivity and trypsinize and count after the eight-hour-incubation. Equalize cell numbers before nuclei extraction.)
- Store the nuclei at -80°C.
- Resuspend nuclei pellet in 1x sample buffer and run acetic acid/urea gel as described.
- Coomassie stain the gel, take a picture and destain.
- Add enhancer solution ("EN^{3}HANCE" by NEN converts -particle energy into photons) and gently rock the gel under the hood at RT for one hour.

- Remove enhancer solution, add cold dH$_2$O and gently rock the gel for another 30 min under the hood at RT to precipitate the fluorescent material inside the gel.
- After the precipitation step has been completed, careflly place the gel onto whatman paper and dry under heat (80°C) and vacuum on a gel-drying-apparatus.
- Place the dried gel against X-ray film (Biomax MR) and expose at -80°C for up to two weeks.

M-19 Running acrylamide gels

stacking gel:	5%	Acrylamide		running gel:	5-15%	Acrylamide
	125mM	Tris pH 6.8			375mM	Tris pH 8.8
	0.1%	SDS			0.1%	SDS
	0.1%	APS			0.1%	APS
	0.1%	TEMED			0.04%	TEMED

3X protein loading buffer:	250mM	Tris
	25%	Glycerol
	5%	SDS
	0.25%	Bromphenolblue

- Prepare stacking and running gel.
- Mix desired amount of protein extract with 3X PLB.
- Boil for 4-5 min at 99°C.
- Spin shortly and leave at RT until loading.
- Load protein samples onto prepared protein gel assembly with a Hamilton pipette and run gel at 80-150V for one to three hours depending on the protein size you wish to detect.

M-20 Running Triton-Acid-Urea (TAU) gels

Usage of Triton/Acid/Urea (TAU) gels offers resolution of post-translationally modified histone isoforms, which could not be separated in conventional SDS PAGE systems due to their similarities in size and charge.

The urea acts as a denaturing agent and acetic acid helps to separate histones on the basis of charge. Addition of a nonionic detergent (Triton) permits the simultaneous resolution of histone variants and isoforms modified post-translationally.

Stacking gel:	0.9M	glacial acetic acid		Acetic acid/urea gel:	0.9M	glacial acetic acid
	0.75%	TEMED			0.5%	TEMED
	6M	Urea			6M	urea
	0.13%	APS			0.13%	APS
	3.3%	Acrylamide			0.37%	Triton
	0.16%	Bisacrylamide			15%	acrylamide
	in H$_2$O				0.1%	bisacrylamide
					inH$_2$O	

1x Sample buffer:	5.8M	urea		Scavenger solution:	2.12M	2-mercaptoethylamine
	0.9M	glacial acetic acid			2.5M	urea
	16%	glycerol			0.87M	glacial acetic acid
	0.2%	methyl green			inH$_2$O	
	4.8%	2-mercaptoethanol				

- Mix components of acetic acid/urea gel and pour into large protein gel apparatus. Remember that shrinking will occur within the polymerisation process. Overlay with H_2O and polymerize one hour.
- Remove layer of H_2O and add stacking gel mixture. Save some stacking before addition of acrylamide/bisacrylamide, to be able to replace shrinked areas. Place a 10-well comb between plates and insert it to the desired position.
- Polymerize for at least two hours at RT.
- Fasten the gel assembly to the apparatus and fill both chambers with 1x running buffer RB (0.9M galcial acetic acid). Connect the power supply in a way that the positive electrode is placed uppermost. Histones will run towards the negative pole!!!
- Preelectrophorese the gel at 130V (constant voltage) until the current no longer falls (4-5 hours). During this time periodically shutt off ther power supply and rinse the wells usind a disposable syringe (with 27-G needle). Also remove any air bubbles that have accumulated at the bottom surface of the gel using a pasteur pipette bent into U-shape.
- Remove RB from both chambers, add water to the top chamber and rinse the wells. Pour the water from the chamber and remove excess water.
- Distribute scavenger solution evenly throughout the wells, slowly add fresh running buffer so that it overlays the scavenger. Also fill lower chamber and continue electrophoresis at 300V for two hours.
- Add fresh RB to both chambers and rinse the wells with a syringe filled with RB.
- Resuspend nuclei pellet in 1x sample buffer and load on acetic acid/urea gel with a Hamilton syringe. (Rinse each well again with a syringe filled with running buffer immediately before loading each sample, to remove any leaching urea.)
- Elerctrophoresis the gel at 200V o/n (about 15 hours) at 4°C.

M-21   Coomassie staining of protein gels

Staining solution:	45%	methanol	Destain solution:	30%	methanol
	10%	glacial acetic acid		10%	glacial acetic acid
	1 g/L	Coomassie R250		inH_2O	
	in H_2O				

- Deassemble protein gel assembly and transfer protein gel to a box filled with staining solution. Incubate for five hours at RT or o/n at 4°C on a rocking platform.
- Remove staining solution wash once with H_2O and add destaining solution. Incubate for five hours at RT or o/n at 4°C on a rocking platform with occasional renewal of destaining solution, until the gel has reached the desired stining intensity.

M-22   Standard Western protocol

For detection of standard protein-antibody detections without requirement for extraordinary sensitivity, this basic western protocol was used. Here polyclonal antibodies against K4-dimethylated histone H3 were bound by a secondary antibody anti-rabbit peroxydase. The ECL mix used after the second antibody binding reacts with the peroxydase group and produces chemiluminescence, which can be detected on a normal Kodak film. Buffers used are described in the section "western star Kit" and "Nuclei extraction" if not otherwise mentioned.

Blocking buffer :	5%	milkpowder		PBS/Tween :	0.1% Tween
	0.1%	Tween			in PBS
	in PBS				

- If nuclei extract is the desired starting material, use nuclei pellet from one 10-cm-dish. Add – NP40 buffer to a concentration of 10^5 cells/µl. Use about 5x 10^5 cells for loading.
- If protein extract is the desired starting material, use desired protein amounts for loading.
- Add 3x protein loading buffer and load on a large Polyacrylamid gel.
- Run o/n 80V at 4˚C (or 4 hours 175V) until the blue front is just leaving the gel.
- Blot for 45 min, 15V in a semi-dry blotter.
- Incubate in blocking buffer for two hours at RT (or o/n at 4˚C).
- Add primary antibody (K4H3 1:1000, Actin 1:2000) in blocking buffer and incubate for one hour at RT.
- Wash three times five min in PBS/0.1% Tween
- Add secondary antibody (anti rabbit 1: 2000) in blocking buffer and incubate for one hour at RT.
- Wash once with PBS/Tween for 10 min, then three times 5 min.
- Mix ECL solutions (Amersham) 1:1 and add to cover the membrane completely.
- Incubate for 5 min, drain on a piece of tissue and expose to X-ray film for 1-20 min.

M-23 Western-Star-Kit (*TROPIX*)
Since TRX2 is a protein with very low abundance in the cell, it is hard to detect it with a standard western protocol. All experiments performed to detect TRX2 with the specific anti-TRX2 were done with the *TROPIX* Western STAR™ protein detection kit.

10x Running buffer :	250mM	Tris	Transfer buffer :	50mM	Tris
	400mM	Glycin		20%	Methanol
	1%	SDS		40mM	Glycine
				0.1%	SDS

Blocking buffer:	0.2%	Casein (content of AppliedBiosystems "Western-*Sta*r™ Kit")
	0.1%	Tween
	in PBS	

10x Assay buffer:	200mM	Tris pH 9.8
	10mM	MgCl$_2$

Substrate solution:	0.25mM	CDP-*Star*® Ready-To-Use (content of "Western-*Sta*r™ Kit")
	1x	Nitro-Block™ enhancer (content of "Western-*Sta*r™ Kit")

- Mix crude protein extracts with 3x protein loading buffer and prepare for loading as described.
- Run a 5% SDS-polyacrylamid-gel for 3 hours at 80V.
- Blot semi-dry at 25 V for one hour.
- Prepare blocking buffer (cook 200 ml PBS in a microwave, take to cold heating bock, switch to 200˚C and stir gently. Slowly (1 min) add 0.4 g casein powder, leave until almost dissolved. Make sure it does not boil! Transfer to 4˚C and keep stirring for about 30 min. Add Tween after it reached RT and store at 4 ˚C)
- Rinse membrane shortly in PBS, then block o/n at 4˚C on a rocking platform.
- Rinse membrane twice, then wash twice five min in PBS/0.1%Tween.

- Incubate for 1 hrs with primary antibody in blocking buffer
- Rinse membrane two times, then wash four times 15 min in PBS/0.1%Tween.
- Incubate for 1 hr with secondary goat anti rabbit alkaline phosphatase conjugate (1:5000) (content of "Western-*Star*™ Kit") in blocking buffer.
- Rinse membrane two times, then wash two times five min in PBS/0.1%Tween.
- Wash two time two min with 1x assay buffer.
- Drain membrane by touching a corner on a paper towel, then place on plastic wrap on a flat surface. Do not let blots dry !
- Pipette 3 ml substrate solution onto the blot and incubate for five min.
- Drain excess substrate solution and place blot in development folder or wrap in plastic. Smooth out bubbles or wrinkles.
- Image blots by exposing them 30 sec to 30 min to X-ray film.

M-24   Creating a TRX2 specific polyclonal antibody

In the process of creating a specific antibody against the mouse TRX2 protein, a region in the N-terminus that shows no homology to any other trx-G family member was choosen. The final peptide spans amino acid 56-96 (nucleotide 2262-2775 of the Trx2 c-DNA) of TRX2 and has no overlap with any of the known protein domains.

Primer pair E+F was used to amplify a PCR-product of about 600 bp from the Trx2 c-DNA clone cF28-1. The EF fragment was cloned BamHI/EcoRI into the expression vector pGex-2TK which caries an N-terminal Glutathione-S-transferase (GST) tag. After verification of the new construct by digesting PstI/NcoI and HincII, the positive mini-prep clones (pGexEF) were transformed into the bacterial expression strain TG1 and BL21.

M-24-1   Induction and expression of gex-EF:
- Grow o/n culture of BL21- and TG1-pGexEF at 37°C.
- Dilute 1:100 in fresh culture medium and continue growing for about 2.5 hours until OD_{600} of 0.5 at 37°C.
- From rest of o/n culture prepare 30% Glycerol stock and freeze at -80°C
- Take 1 ml, spin down pellet, add 50 µl H_2O and freeze at -20°C
- Induce rest of the culture with IPTG 0.5mM transfer to 30°C and continue growing for about 3 hours until you reached an OD_{600} of 1.
- Take 1 ml, spin down pellet, add 100 µl H_2O and freeze at -20°C
- Add protein loading buffer and load 20 µl of induced and un-induced sample on a 15% polyacrylamid protein gel and run at 100V for one hour.
- Coomassie stain the gel, transfer onto whatman paper and dry in a vacuum gel dryer for 30 min.

The fusion protein gex-EF has an expected molecular weight of 45.2 kDa (411 amino acids). In Figure M-2, a band of the expected size is visible in the induced sample of both expression strains, that is missing in the un-induced sample. EF-Gex is therefore expressed in both TG1 and BL21.

M-24.2   Purification of gex-EF

M-24.2.1   Induction

- Grow 4 x 1L o/n culture of BL21 pGexEF at 37°C.
- Dilute 1:50 in fresh culture medium and continue growing for 3 hr to OD 0.78 at 37°C.
- Chill to 18°C on ice (~15min)
- Induce with 0.2 mM IPTG + 0.2 mM ZnSO4 for 7 hours at 18°C.
- Freeze as pearls after resuspending in 1x PBS

M-24.2.2   Protein extraction

High Salt Lysis Buffer:

1.5%	N-lauroyl-sarcosine
1x	protease inhibitors
1mM	EDTA
1.25M	NaCl
in PBS	

Elution buffer:

50mM	HEPES pH 8
300mM	NaCl
0.5mM	DTT
0.05%	Tween20
10mM	reduced glutathione

(adjust to pH 8 with NaOH after adding the reduced glutathione from a 250mM stock in H2O)

Storage buffer:

		Column buffer:		
20mM	HEPES ph 7.5		0.25M	NaCl
150mM	NaCl		0.05%	Tween-20
0.05%	Tween20		0.5mM	DTT
20%	Glycerol			

- Grind the frozen pearls (cells) to a fine powder under liquid N_2 using a mortar and pestle (pre-cooled with N_2). The ground cells can be stored frozen at -80° C.
- Thaw the ground cells by adding 5 volumes of high salt lysis buffer. If ground cells are at liquid N_2 temperature allow them to warm up until they are just starting to thaw at the edges before adding the lysis buffer. Stir at once with a spatula to break up clumps. Add a stir bar and stir in the cold room for a few minutes (15-30 min).
- Sonicate for 6 x 10 sec pulses (with at least 20 sec pauses between) on wet ice to shear DNA. Use the Branson Sonifier 450D at 70% amplitude with the 10 mm flat tip immersed 1-2 cm below the surface of the liquid.
- Centrifuge extract in Beckmann TYPE 45Ti @ 38, 000 rpm, 4°C for 45 min. Fill tubes to at least 3/4 full adding buffer if there is not enough extract.
- Transfer the supernatant to a 100 mL graduated cylinder.
- Add 5 mM DTT (final concentration) to the supernatant.

M-24.2.3   Affinity chromatography
- Wash 3 ml glutathione sepharose (Pharmacia 17-1756-01; 4 ml of slurry contains ~ 3 ml of packed beads) in a 50 ml conical tube three times with 25 ml of column buffer. Sediment resin between washes using the Megafuge 3SR centrifuge; 4000 rpm for 2 min. Pour off the wash buffer taking care not to discard any beads.

- Add high speed supernatants of cell extract to these washed beads and rotate end-over-end in the cold room for 30min. Sediment the beads.
- Wash the beads once with 50 ml of column buffer, then sediment the beads and discard the supernatant.
- Use a small amount of buffer with a plastic pasteur pipet to transfer all the beads into a disposable column. Wash with 20 CV of Column Buffer With detergent and then with 5 CV of Column Buffer without detergent. Allow all of the buffer to drain through the column.
- Elute fusion protein with Elution Buffer containing 10mM reduced glutathione.
    - o to collect a fraction:
    - a) Add 500 µl of elution buffer to the drained bed
    - b) Collect the flow-through into a 96-deep well plate
    - c) Repeat steps a) and b) 25 times!
    - d) Stop the flow and quickly determine the peak fractions using the Bradford assay. Collect additional fractions if the peak is not yet eluted from the column.
- Pool the peak fractions (up to a total of 2.5 ml) then desalt them into storage buffer or desalt over desalting column on biocad (Pharmacia Hiprep 26-10) pool sample up to 15ml.
- Measure the protein concentration of this desalted-pool by A280 and also by the Bradford assay. Then add glycerol to 20%, fast freeze in liquid N_2 and store @ -80º C.

The purified peptide was sent for polyclonal antibody production in two rabbits (3853g and 3854g). Monospecific IgGs were isolated against the original antigen EF-Gex. (BioGenes)

To test specificity of the newly produced mouse TRX2 antibody, equal concentrations of protein extracts from TAP tagged TRX2 ES cells, wt ES cells, ES cells heterozygous for the trx2 k.o, and ES cells homozygous for the trx2 k.o., were separated on a 5% polyacrylamid protein gel and probed with anti-mTRX2 (3854g 1:300). The result is displayed in figure M-3. The band detected by anti-TRX2 in TAP-Trx2 cell extracts about 300kDa. The band detected in wt and Trx2 heterozygous extracts is slightly smaller since the TAP tag of about 10 kDa is missing. The band intensity in the wt sample is reduced to half in the +/- sample and completely absent in the -/- sample. It was thus concluded that anti-mTRX2 is specific for TRX2. It does not detect other closely related trx-G family members like MLL.
Colaborators (Meisterernst et al.) have tested affinity purified IgG derived from both rabbits and found that only 3853g works satisfactory in ChIP experiments. Western experiments performed by me and other lab members also confirmed 3853g as the antibody with highest affinity and specificity to TRX2.
Both antibodies have been aliquoted in 30% glycerol and stored at -20°C.
<u>3853g</u> (400 µg/ml) - use 1:4000 dilution   (240µg/ml) – use 1:2000 dilution
<u>3854g</u> (30 µg/ml) - use 1:300 dilution   (200µg/ml) – use 1:2000 dilution

Two additional rabbits (4676 and 4675) have been injected with the EF-gex antigen and bled for antiserum extraction. Aliquots are stored at -80°C.
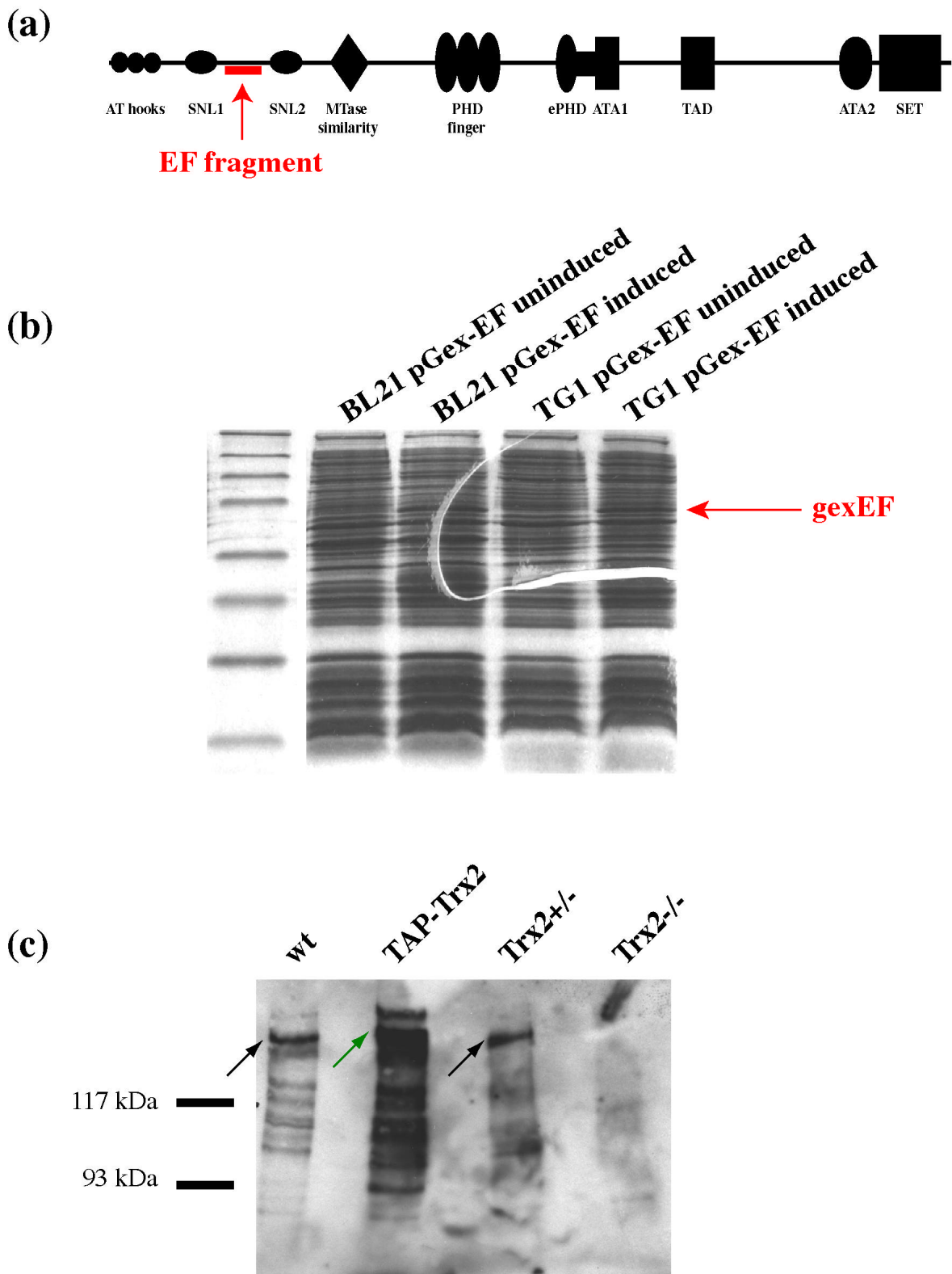
(a) AT hooks · SNL1 · EF fragment · SNL2 · MTase similarity · PHD finger · ePHD · ATA1 · TAD · ATA2 · SET

(b) BL21 pGex-EF uninduced · BL21 pGex-EF induced · TG1 pGex-EF uninduced · TG1 pGex-EF induced · gexEF

(c) wt · TAP-Trx2 · Trx2+/- · Trx2-/- · 117 kDa · 93 kDa

Figure M-3: Specific TRX2 antibody (anti-TRX2) raised against the EF fragment
(a) Loaction of the Trx2 specific EF fregament within the TRX2 protein.   **(b)** Protein expression profile of pGex-EF in the expression strains BL21 and TG1 before and after induction with IPTG. In both expression strains upon induction a band of the expected size (45.2 kDa) becomes visible that is invisible in the uninduced state.   **(c)** Western on crude protein ES cell extracts using the new anti-TRX2 antibody. The 280 kDa TRX2 band (indicated by black arrow) is detected in wt and Trx2 heterozygous (+/-) ES cells lower bands are TRX2 degradation products. In TAP-Trx2 ES cells that carry a TRX2 20 kD N-terminal TAPtag, anti-TRX2 recognizes the corresponding 300kDa band (indicated by green arrow) while no signal is visible in ES cells deficient for Trx2 (-/-).

WORKING WITH EMBRYONIC STEM (ES) CELLS

M-25   Preparing Mouse Embryonic Fibroblast cells (MEFs, feeders)
All ES cell experiments were performed in feeder dependent 129 cell lines. To maintain their pluripotency in long-term culture, these ES cell lines should be grown an monolayers of mitotically inactivated fibroblast cells (Thomas and Capecchi, 1987; Leighton et al., 1995). Feeder cells are derived from mouse embryonic fibroblast tissue, expanded up to maximally 15 cell divisions and then inactivated by mitomycin treatment to prevent further cell divisions.

feeder -media: 10%   Fetal Calf Serum
                   100 u:µl/ ml   penicillin/streptomycin
                   2mM   L-glutamine
                   in DMEM high glucose

For cultivation feeder cells are thawn onto 0.1% gelatine coated Falcon plates and incubated at 37 ˚C, 5% CO_2. Cells were passaged one day after they reached 100% confluency (roughly every three days) by trypsinization. The original vial purchased by *Medicore* was expanded to in total 21 15-cm-dishes before a 2-2.5 hour treatment with 10 µg/ml mitomycin. Those mitomycin treated feeder cells were frozen and upon need plated in 100% confluency (about 10^6 cells) one day before seeding the ES cells.

M-25.1   Expanding MEF cells

- Thaw original feeder vial onto gelatine coated 10-cm-dish. After one day, the plate should be 100% confluent. Change media and leave one more day in the incubator.
- Remove media and rinse plates with 1x PBS.
- Add 1x trypsin/EDTA (2 ml on a 10-cm-dish, 7 ml on a 15-cm-dish) and leave the plate at 37˚C for 2 min. Immediately add feeder media to inactivate the trypsin.
- Pipet the suspension up and down to remove cell clumps.
- Transfer the suspension into a 15-ml-falcon tube containing media and spin 1000 rpm for 5 min at RT (*Multifuge* 3 S-R, rotor 6445).
- Aspirate supernatant and resuspend cells in media. Then split 1/3 and plate on new gelatine coated dishes.
- Repeat this procedure until you reach 21 times confluent 15-cm-dishes.

M-25.2   Freezing MEF cells

2x freezing media: 50%   Fetal Calf Serum
                       20%   DMSO
                       in DMEM high glucose

- Trypsinize cells from a confluent 15-cm-dish as described.
- After spinning down in media, resuspend cells from one plate in 0.5 ml media and cool down on ice for 5 min.
- Add 0.5 ml 2x freezing media and trasfer to 1-ml freezing vials then freeze at -80˚C.
- Transfer to liquidN the next day. This vial contains enough feeder cells to cover five 10-cm-dishes.

M-26   Culturing mouse ES cells

Mouse embryonic stem (ES) cells are totipotent cells derived from the inner cell mass of 3.5 day blastocysts. They can be modified and reintroduced into blastocysts, giving rise to manipulated chimeric animals. (Thompson et al. 1989)

If they are held under certain growth conditions, they can also differentiate to various cell types. To avoid this, LIF serum is added to the ES medium. It suppresses differentiation, so that the silencing of genomic regions during the differentiation process is prevented.

ES cell media: 15%   Fetal Calf Serum
                  100 u:µl/ ml   penicillin/streptomycin
                  100µM   non-essential amino acids
                  1mM   sodium acetate
                  1µM   -mercaptoethanol
                  2mM   L-glutamine
                  600 U/ml   LIF "ESGRO"
                  in DMEM high glucose

For cultivation ES cells were thawn onto feeder coated 10-cm Falcon plates and incubated at 37 °C, 5% CO_2. After ES cells are thawed it is recommended to passage them every second day by trypsinization. Reseeding should be calculated in a way that the cells reach 100% confluency after 48 hours in culture.

For long-term storage ES cells are kept in liquid nitrogen in a cell density of 5x 10^6 cells in freezing medium containing DMSO as a cryoprotectant. Since DMSO is harmful to cells it should contact ES cells as short as possible before and after freezing.

M-26.1   Harvesting ES cells

- Remove media and rinse plates with 1x PBS.
- Add 1x trypsin/EDTA (2 ml on a 10-cm-dish) and leave the plate at 37°C for 5 min.
- Pipet the suspension up and down to remove cell clumps.
- Transfer the suspension into a 15-ml-falcon tube containing ES media and spin 1000 rpm for 5 min at RT (*Multifuge* 3 S-R, rotor 6445).
- Aspirate supernatant and resuspend cells in 3-8 ml of ES media. Then plate out an appropriate fraction on a new dish. A rough estimate is that a confluent 10cm dish should yield 1-4 x 10^7 cells and approximately 1-5 x 10^6 cells should be seeded on a 10cm dish in 10 ml media.

M-26.2   Freezing ES cells

2x freezing media: 50%   Fetal Calf Serum
                      20%   DMSO
                      in DMEM high glucose

- Trypsinize cells from a confluent 10-cm-dish as described.
- After spinning down in media, resuspend cells from one plate in 2 ml ES media and cool down on ice for 5 min.
- Add 2 ml 2x freezing media and seperate into 1-ml freezing vials then freeze immediately at 80°C.
- Transfer to liquidN the next day.

M-27 Preplating

For all western experiments it was desirable to harvest a pure colony of targeted ES cells without contamination of wt feeder cells. In order to obtain this purity, ES cells cultured on feeders were pleplated. This preplating event removes feeder cells to about 90%.

- For 10cm-dish add 3 ml trypsin/EDTA remove and add 3 ml again.
- Leave plate in the incubator for 15 min.
- Add 7 ml ES media and strongly pipet up and down to disrupt cell colonies.
- Spin 5 min at 1000rpm and remove supernatant.
- Resuspend in 10 ml ES media and transfer the cells onto a gelatine uncoated tissue culture dish.
- Leave in the incubator for 20-30 min
- Carefully pipet off the supernatant and transfer ES cells (90% feeder-free) onto a new 0.1% gelatinized dish.

M-28 Electroporation

To transform DNA constructs into ES cells the electroporation method was applied. For electroporation it is best to use cells that are passaged twice after thawing.

- Trypsinize cells from a 10-cm-dish and make sure that it is really a single cell suspension!
- After spinning in media for 5 min, aspirate the media, and resuspend the cell pellet in 5 ml PBS. Take an aliquot for counting and spin 1000 rpm at RT for 5 min.
- During the centrifugation step, count the actual number of cells on a haemocytometer
- Aspirate the supernatant and resuspend cell pellet in an appropriate volume of PBS, so that the final volume of 800µl for the electro execution contains 10^7 cells.
- Use 40 µg linearized targeting plasmid (for Trx2-/- targeting use Fe13-lacZ-hygro, for EYFP-Trx2 targeting use X2.2-EYFPneo-trx2) in 50 µl PBS. Put linearized DNA into the gene pulser cuvette (0.4 cm electrode gap) and add 800µl cell suspension.
- Set Bio-Rad electroporator at 240V, 500µF and pulse the cells (the pulse time should be between 5 and 6s.
- Transfer the electroporated cells to 60-80 ml media and distribute cell suspension onto 6-8 gelatinized 10-cm-dishes.

M-29. Transformation
M-29.1   Stable transformation

Stable transformatuion in this thesis was applied to target ES cells for the Trx2 k.o and the EYFP-Trx2 fusion.
- Thaw ES cell clone (for Trx2-/- targeting use E14 Trx2+/- clone 2-88, for EYFP-Trx2 targeting use E14 wt clone) onto a feeder coated cell culture dish.
- After two passages perform electro-transformation as described.
- Plate transformed cells on six 10-cm feeder-coated dishes.

- For Trx2-/- targeting: After 40 hrs change media and add 200 µg/ml hygro, 250 µg/ml G418 selection. For EYFP-Trx2 targeting: After 24 hrs change media and add 250 µg/ml G418 selection
- Incubate for 8-10 days with occasional change of selection media.
- Pick about 96 undifferentiated colonies into feeder containing 48-well-dishes.
- After two days, trypsinize and transfer onto new feeder containing 48-well-dishes.
- After another two days, split each clone in two: One third stays in the 48-well-dish. Cool 5 min on ice then add 2x freezing media and freeze at -80°C. Transfer the reamining two thirds trypsinized cell suspension into gelatinized 24-well-dishes containing 800 µl ES selection media. After 3-5 days these wells will be confluent and cells can be used for DNA extraction.

M-29.2   Transient transformation

Transient trasnformation in this thesis was applied to introduce the Cre recombination plasmid into EYFPneo-trx2 targeted ES cells.

- Thaw EYFPneo-trx2 clone #22 and passage twice before collecting 10^6 cells for electroporation.
- Use 40 µg unlinearized McCre plasmid resuspended in 50 µl 1x PBS and perform electroexecution as described.
- After electroporation seed 5-50 x 10^3 cells per feeder coated plate and incubate for 7-9 days with occasional media change (no selection !).
- Pick about 96 undifferentiated colonies into feeder containing 48-well-dishes.
- After two days, trypsinize and transfer onto new feeder containing 48-well-dishes.
- After another two days, split each clone in three: One third stays in the 48-well-dish. Cool 5 min on ice then add 2x freezing media and freeze at -80°C. One third is transferred into G418 containing media to check for Cre mediated deletion of the neomycin selection marker. Transfer the reamining one third trypsinized cell suspension into gelatinized 24-well-dishes containing 800 µl ES media. After 3-5 days these wells will be confluent and cells can be used for DNA extraction.

M-30 Propidium Iodine (PI) staining of ES cells

staining solution:	1.8M	NaCl	
	0.7M	MgCl2	
	750 µM	PI	
	1M	Tris, pH 7.5	
	2 mg/ml	RNAseA	(add freshly before use)

- Grow ES cells on gelatine coated 10-cm-dishes for two passages.
- Collect cells by trypsinisation and resuspend about 10^6 cells in 5 ml PBS.
- Centrifuge 5 min at 200 x g
- Prepare fixing solution by filling 15-ml-falcons with 4.5 ml 70% ethanol. Keep tubes on ice.
- Using a pasteur pipette thoroughly resuspend cells in 0.5 ml PBS. (It is important to archieve a single cell suspension!)
- Transfer the cell suspension into the tube containing 70% ethanol and fix o/n at 4°C.
- Centrifuge the ethanol-suspended cells 5 min at 200 x g. Decant ethanol thoroughly.

- Suspend cell pellet in 1 ml PI satining solution and incubate 30 min at RT.
- Set up and adjust the flow cytometer for excitation (488 nm argon ion laser) with blue light and detection of PI emission at red wavelengths (long pass >600 nm filter).
- Measure cell fluorescence in the flow cytometer. Use the pulse width-pulse area signal to discriminate between G2 cells and cell doublets, and gate out the latter.

M-31 Proliferation assay
- Cells from each clone were freshly thawed and expanded to 2x 10-cm-plates on feeders
- Preplate confluent dishes and count. Dilute in media to a rate of 10^5 cells/ml.
- Seed each clone in triplicates into gelatine-coated- and feeder-containing-6-well-dishes (2ml = 2x 10^5 cells/well). Prepare this set-up five times (once for every day of counting).
- One day after plating trypsinize normally and count cells in a hemocytometer. For the "split and count" experiment reseed these cells again onto the same 6-well dish.
- From now on count one set-up of plates every day. For the "split and count" experiment count and reseed same cells every day. On day three transfer onto 5cm-dishes containing gelatine or fresh feeders respectively, otherwise use same dish for reseeding.
- For evaluation of cell numbers take the average of triplicate counts for every time point and display summary in graphics.

M-32 ES-colony methylene blue staining

staining solution: 1% methylene blue
                         in ethanol

- Plate 10^4 cells of wt, +/- and -/- clones in quadruplicates into 6-well-dishes.
- Grow for eight days with occasional media change.
- Remove media, wash with PBS and fix cells for 5 min in ethanol.
- Remove ethanol and replace with staining solution.
- Incubate for 10 min at RT, then remove staining solution and wash with PBS or ethanol for an optimal visualization of the colonies.

M-33 Apoptosis assay
Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular weight DNA fragments as well as single strand breaks ("nicks") in high molecular weight DNA. Those DNA strand breaks can be identified by labeling free 3'OH termini with modified nucleotides in an enzymatic reaction. The Kit performs in three consecutive steps:
1. Labeling of DNA strand breaks, by Terminal deoxynucleotidyltransferase (TdT), which catalyzes polymerization of labeled nucleotides to free 3'-OH DNA ends in a template-independent manner (TUNEL reaction).
2. Detection of incorporated fluorescein by anti-fluorescein antibody Fab fragments from sheep
3. Analysis under fluorescence microscope.

fixation solution: 4%   paraformaledehyde
                              in PBS

permeabilisation solution:	0.1% Triton X-100	digestion buffer:	3U/ml DNAseI
	0.1% sodium citrate		50mM Tris-HCl, pH 7.5
	in H$_2$O		10mM MgCl$_2$
			1 mg/ml BSA

label solution: nucleotide mixture in reaction buffer (content of "*In Situ* Cell Death Detection Kit")

enzyme solution: Td from calf thymus in storage buffer (10x) (content of "*In Situ* Cell Death Detection Kit")

- Grow ES cells in 24-well-plate on coverslips.
- Wash with PBS.
- Fix air-dried samples with freshly prepared fixation solution for one hour at RT.
- Wash with PBS.
- Incubate in permeabilisation solution for two min on ice.
- For positive control incubate fixed and permeabilized cells with DNAseI in digestion buffer for 10 min at RT to induce DNA strand breaks, prior to labeling procedures.
- Prepare TUNEL reaction mixture by adding 50 µl enzyme solution to 450 µl label solution. Mix well to equilibrate components.
- Rinse slides twice with PBS.
- Take coverslips out of the well, dry by touching a paper towel and place upside down onto a drop of 50 µl TUNEL reaction mixture.
- For negative control incubate fixed and permeabilized cells in 50 µl label solution (without TdT) instead of TUNEL reaction mixture.
- Incubate for one hour at 37 °C in a humidified atmosphere in the dark.
- Dip coverslips 3x in PBS
- Samples can be analyzed in a drop of PBS under a fluorescence microscope at this state. Use an excitation wavelength in the range of 450-500 nm and detection in the range of 515-565 nm (green).

M-34   Blastocyste injection of ES cells

Blastocyts were collected from the uterus of four days pregnant C57/BL6 females. Targeted ES cells (Trx2-/- clone #13; EYFPtrx2 clone #70 and #95) grown to confluency on feeder cells were injected into the inner cell mass and trasferred back into the uterus of pseudopregnant foster mothers on the fourth day of pregnancy.

All ES cell injections were performed by the EMBL Transgenic service, according to standard procedures.

WORKING WITH TRANSGENIC MICE

M-35 Genotyping

Pups were weaned at three weeks of age to take a 1-cm sample of the tail tip for genotyping. Tail-DNA extaction was performed according to the protocol in M-11. Gentyping was based on a PCR strategy (for further details please refer to the results section)

M-36 Whole mount embryo lacZ staining

<u>fixing solution</u>:   4% Paraformaldehyde   <u>transport solution</u>:   PBS/ 0.4% BSA

<u>staining solution</u> :

0.01%	Sodium-Deoxycholate	
0.02%	NP40	
2 mM	MgCl$_2$	
8 mg	Spermidin-trihydrochloride	
fill to 30 ml with PBS. Stable at 4°C for several weeks.		
10 mM	Ferrocyanide	add just before use
10mM	Ferricyanide	add just before use
2 mM	Xgal	add just before use

<u>sucrose solution</u>:   30 % sucrose in PBS

- Dissect embryos at RT in transport solution. Transfer all the embryos into fixing solution (5-cm-dish) and incubate on ice for 30 min to 1 hour (depending on developmental stage of the embryo).
- Transfer into a 10-cm-dish containing cold PBS to wash.
- Transfer embryos into a 5-cm-dish containing freshly prepared staining solution.
- Incubate at 37°C o/n. To avoid evaporation and drying out of the embryos place dishes into a box layed out with wet tissue.
- To take pictures wash embryos in PBS twice.
- If it is intended to make sectionings of the embryos, incubate them in sucrose solution o/n at 4°C. Sucrose also transluscence tissue, so that the blue staining becomes clearer.

M-37 Kryosectioning and staining
M-37.1 Slide preparation
- Dissolve gelatine in H$_2$O to make a 0.5% solution
- Coll the solution to RT and add chromium potassium sulfate 0.05%.
- Cool the solution to 4°C on ice. (Use this subbing solution immediately)
- Wash glass slides twice for 10 min in ddH$_2$O,
- then twice for 5 min in subbing solution.
- Dry under the hood o/n.
- Keep protected from dust at RT for up to three months.

M-37.2   Embryo preparation and sectioning:

- Dissect embryos of desired developmental stage in cold transport solution.
- Immediately transfer to embedding molds filled with OCT and freeze samples on a block of dry ice.
- Store frozen embryos at -80°C until sectioning.
- Cut 50-µm-sections on a kryostat mashine. Transfer every 10th section of an embryo to a gelatine subbed glass slide and air-dry, to finally have about 20 sections per embryo.

M-37.3   Fixation and staining:

solution A:	0.2%	gluteraldehyde		solution B:	2mM	$MgCl_2$
	5mM	EGTA, pH 7.3			0.02%	NP40
	2mM	$MgCl_2$			in PBS	
	in PBS					

solution C:	1 mg/ml	X-Gal (dissolved in DMSO)
	4mM	potassionferrocyanide
	4mM	potassionferricyanide
	in solution B	

- Fix sections in solution A for 15 min at RT.
- Wash three times 10 min in solution B at RT.
- Stain sections in solution C o/n (up to 20 hours) in the dark.
- Wash three times 10 min with solution B, wash once quickly with PBS.
- Fix 5 min in PFA (4% in PBS).
- Wash twice 5 min with PBS.
- Counterstain sections with eosin (1/10 diluted in PBS) for 1 min 30 sec.
- Dehydrate in 95% ethanol, dip slides eight times in the same 95% ethanol, then dip them again eight times in 100% ethanol.
- Fully dehydrate one min in 100% ethanol.
- Mount in Erkitt solution and cover with coverslip. Store at RT.

R. Results

R-1   *TRX2 KNOCK-OUT IN ES CELLS (TRX2-/-)*

Indications from former publications suggest for TRX2 a role in differentiation and transcriptional control on the chromatin level (see introduction). Since mouse embryonic stem (ES) cells are a powerful and manageable tool for in vivo cell culture experiments, they can be used for in vitro differentiation assays that lead to faster results than mouse, it was decided to create a homozygous knock out of TRX2 in the ES-cell model system.

In order to create an ES cell line that carries a homozygous knock out (k.o.) for the Trx2 gene, work began with modifying an existing Trx2 k.o. cassette. This cassette came from a previous project in our lab to create a Trx2 k.o. mouse.

R-1.1 Cloning of targeting construct pFe13lacZ-hygro

The targeting construct Fe13lacZneo that had been successfully used by Frank v.d. Hoeven to create an ES cell line heterozygous for the Trx2 knockout is depicted in figure 1. The lacZ gene codes for the enzyme -galactosidase that produces a dark blue color upon incubation with its precipitating substrate X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) and is often used in reporter assays. The neo gene conveys resistance to kanamycin in prokaryotic cells and to geneticin (G418) in eukaryotic cells.

After homologous integration of the construct into the first intron of the endogenous Trx2 genomic locus, the splice acceptor (SA) ensures splicing of exon one to lacZneo. Then the polyA signal 3' of lacZ-neo truncates the Trx2 transcript so that the targeted ES cells do not express TRX2 protein, but display lacZ staining and G418 resistance expressed from the Trx2 promoter.

To achieve a complete Trx2 knockout, the second Trx2 allele in the diploid mouse genome had to be targeted. To reach that homozygous state, two different methods were tested: Heterozygous ES cells containing one copy of the G418 resistance gene were held
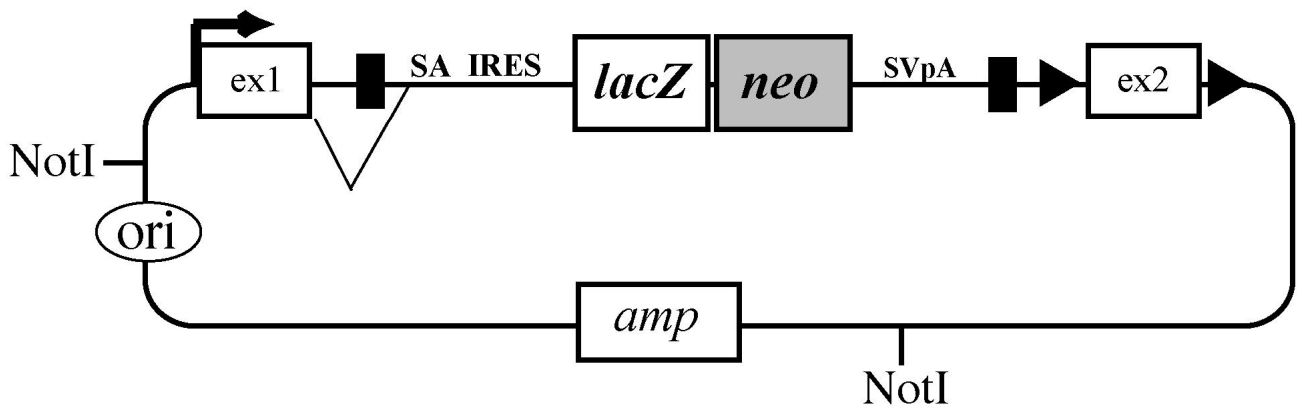
Figure. 1: Features of pFE13lacZneo-Trx2 targeting construct.

- Origin of replication (ori), and ampicillin resistance (amp) allows propagation in E.coli
- Trx2 endogenous promoter (indicated by black arrow), exon1 (Ex1) and intron2 serve as homology arms for homologous integration of the NotI linearised construct into the endogenous Trx2 locus.
- Trx2 transcription is terminated by the SV40 polyadenylation signal (SVpA) resulting in a Trx2 knock-out since only Ex1 fused to lacZ/neo is being expressed.
- Splice acceptor (SA) ensures fusion of Ex1 to the lacZ/neo cassette.
- Internal Ribosome Entry Site (IRES) ensures efficient translation of the lacZ/neo gene products.
- LacZ/neo expression is regulated by the endogenous Trx2 promoter and therefore mimics the Trx2 expression pattern.
- Black rectangles indicate FRT sites (target sites for Flp-recombinase), black triangles indicate loxP sites (target sites for Cre-recombinase). Flp-mediated recombination exises the complete k.o. cassette reverting back to the wt situtation. Subsequent Cre-mediated recombination leads to deletion of exon two, an event that destroys the ORF and thus creates again a knock-out allele.

in increasing concentrations of G418. After massive cell death the ES cells might react to that selection pressure by duplicating the G418 resistance gene through mitotic recombination (Nelson et al., 1989; Potter at al., 1987) or chromosomal duplications (Wasmuth and Hall, 1984; Campbell and Worton, 1981) so that surviving ES cell clones would carry the targeting construct also on the second allele of Trx2 (Mortensen et al., 1992). Unfortunately heterozygous Trx2 ES cells were able to tolerate even selection with up to 2000 µg/ml G418 without significant cell death. Thus it had to be concluded that the promoter driving the G418 resistance gene in the Fe13lacZneo construct was simply too strong. Also in several publications where this method has been successfully applied for loss of heterozygousity, a mutated version of the G418 resistance gene was used (Lefebvre et al., 2001).

The second approach to reach homozygousity included targeting of the second allele by a second round of electroporation. In order to be able to select for the integration of two different k.o. cassettes into both Trx2 alleles, the neomycin gene in Fe13lacZneo was replaced by the gene conveying hygromycin resistance. Doubly targeted cells would then be double resistant to both G418 and hygromycin selection. To achieve the G418→hygromycin replacement the ET/Red cloning technology (Zhang et al., 1998; Muyrers et al., 1999) was applied. The cloning strategy for Fe13lacZhygro is described in figure 2. The oligos EThygro-up and EThygro-dw consist of a 3' PCR primer sequence and a 5' 50bp homology arm sequence (figure 2c). A PCR reaction on the hygromycin template pC-DNA$_3$ with those oligos yielded a product (PCRhygro) containing the hygromycin gene ORF flaked by two homology arms that reconstitute the exact sequence of the desired integration site in Fe13lacZneo. DpnI, an enzyme that specifically recognizes only methylated restriction target sites and not the unmethylated PCR product was used to remove residual template DNA. After precipitation (to concentrate the DNA solution), the linear product PCRhygro and recipient plasmid pFe13lacZneo were coelectroporated into electro-competent and arabinose-induced *E. coli* DH10B/pSC101/BAD/YZA cells. This bacterial strain expresses the ET/Red recombinases recE, recT and red under control of the arabinose-inducible BAD promoter (kindly provided by Zhang -Genebridges-). During the following 70-min-incubation at 30 °C, these enzymes mediated the replacement of the neomycin coding
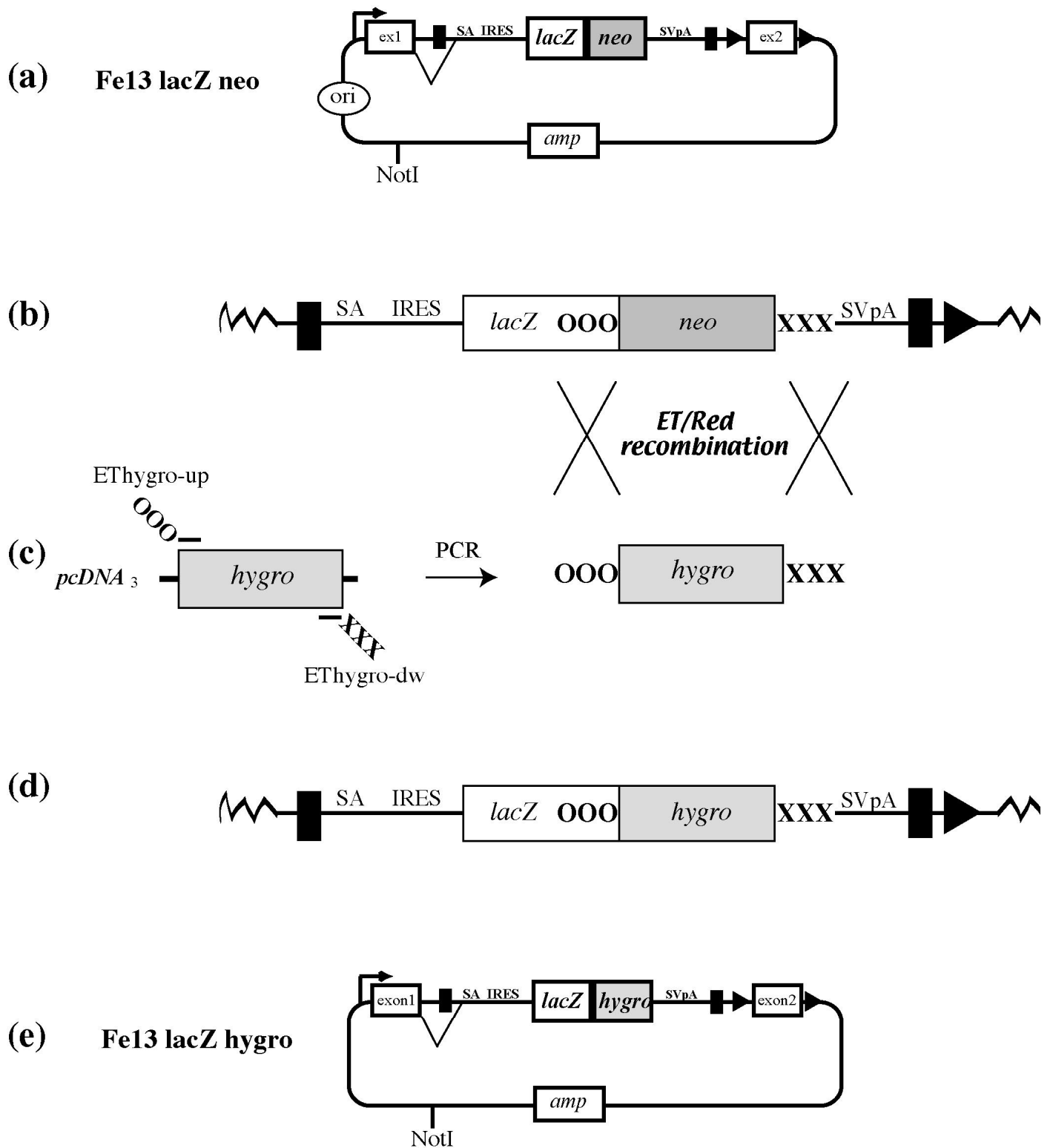
Figure. 2: **Cloning strategy for conversion of pFE13lacZneo to pFE13lacZhygro.**

(**a**) Targeting construct pFE13lacZneo as described in Fig.1. (**b**) Close up of the LacZ/neo region pFE13lacZneo. OOO= 5' homology arm sequence for ET recombination (upstream), XXX= 3'homology ar sequence for ET recombination (downstream). (**c**) PCR amplification from the hygromycin templa pcDNA3 using the oligos EThygro-up and EThygro-dw. The resulting PRC product contains the hygromyc ORF flanked by the 5' and 3' homology arms for ET recombination. The ET/Red recombination event indicated by two black crosses. (**d**) Close up of the LacZ/neo region in pFE13lacZhygro after the ET/R recombination event . (**e**) Newly created targeting construct pFE13lacZhygro.
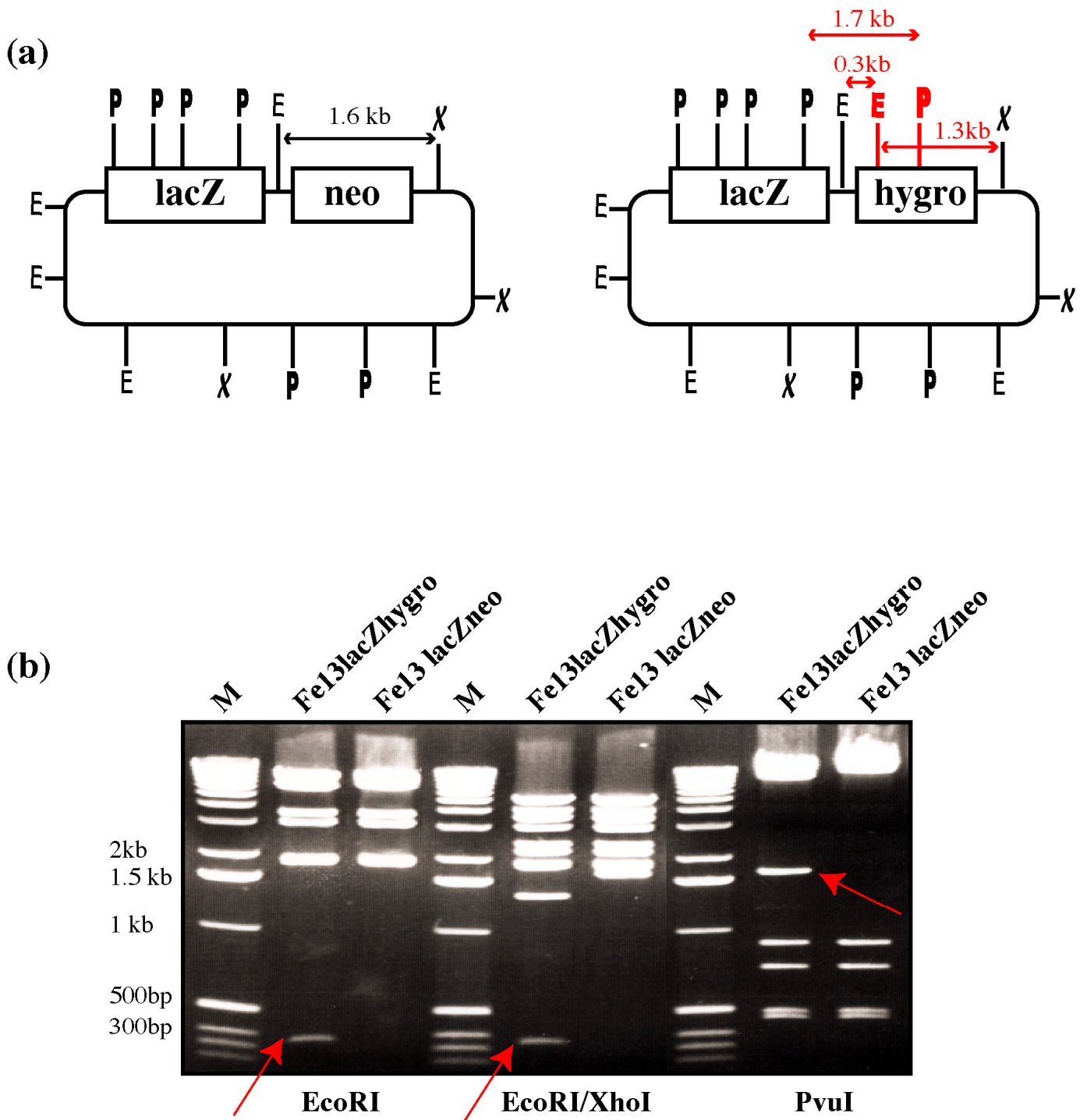
Figure 3: Mini digestion confirmes cloning of Fe13lacZhygro

(a) Graphical display of relevant restriction sites EcoRI (**E**), XhoI (**X**) and PvuI (**P**) in Fe13lacZ neo (left) and Fe13 lacZhygro (right). Restriction sites in which neo and hygro differ are indicated in red. Relevant restriction fragments are indicated by two-sided arrows in black for Fe13lacZneo and in red for Fe13lacZhygro. **(b)** EcoRI, EcoRI/XhoI and PvuI restriction pattern: Additional EcoRI site in hygro creates 300bp fragment in EcoRI and EcoRI/XhoI digest, additional PvuI site creates 1.7 kb fragment in PvuI digest (relevant fragments are indicated by red arrow) M marks the kb-marker lane. Fragment sizes are indicated left of the digestion pattern.

sequence for the hygromycin coding sequence in the targeting cassette of Fe13lacZneo via the homology arms in PCRhygro (figure 2d);(for detailed description of the Red/ET recombination process, see method section M-9). The ET expression plasmid was lost upon o/n incubation of the plated bacteria at 37°C because of their temperature sensitive origin pSC101.

Mini prep digestions with EcoRI, PvuI and EcoRI/XhoI confirmed the desired replacement of the G418- with the hygromycin- selection gene in pFe13lacZhygro (figure 3).

R-1.2 Creation of a homozygous Trx2 knock-out ES cell line

In order to achieve the complete k.o. for Trx2, the second targeting construct Fe13lacZhygro had to be stably introduced into an ES cell line that already carried the heterozygous Trx2 mutation. Organization of the genomic Trx2 gene locus is displayed in figure 4. The genomic subclone Fe13 spans the promoter region and the first two exons of the Trx2 genomic locus (figure 4c). The targeting constructs Fe13lacZhygro and Fe13lacZneo contain the respective k.o cassette integrated into intron one (figure 4d and e). Homologous integration of both k.o. constructs into the genomic locus is mediated by about 8 kb upstream promoter and exon 1 sequences and 6 kb downstream intron one, exon two and intron two sequences of Fe13 (in figure 4d and e indicated by gray shaded areas).

For stable transformation of pFe13lacZhygro, the plasmid was linearized and electroporated into the Trx2-heterozygous ES cell clone 2-88. After double selection in 200 µg/ml hygromycin and 250 µg/ml G418, stable colonies were picked and grown up for genomic DNA extraction. To confirm desired stable integration of the cassette into the homologous locus, a southern strategy was developed (in figure 5d clone #6 and #13 are displayed). Probing with a hygromycin fragment confirmed the presence of the targeting construct lacZ-hygro in the genome. EcoRI digestion revealed a fragment of the expected size of 12 kb. Correct integration for the 5' side was tested by PacI/SalI digestion of genomic DNA and probing of the southern blot with the X4 fragment that anneals to a 650bp-region 5' of exon one and therefore outside the targeting cassette. In
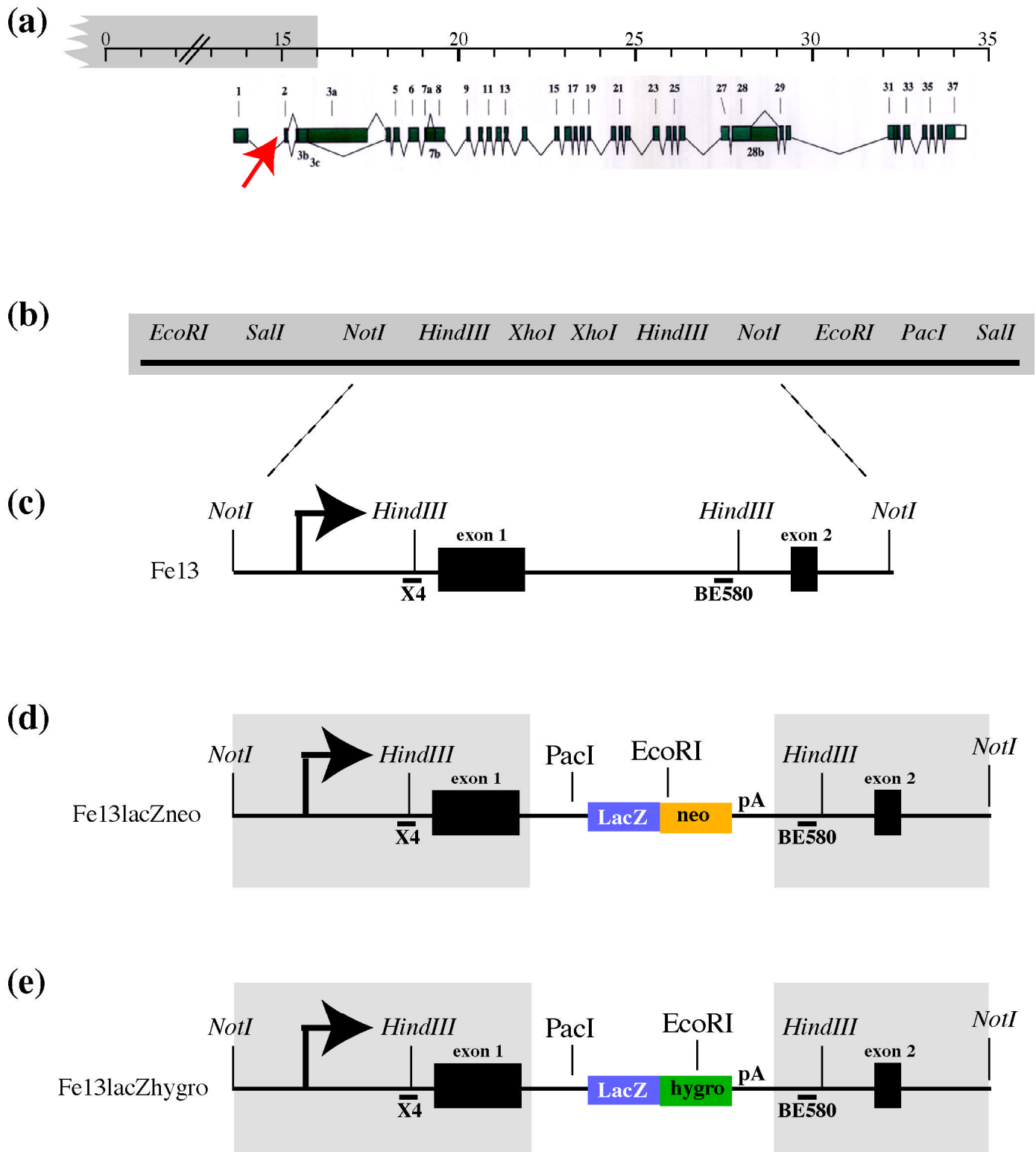
Figure. 4: Genomic organization and knock out strategy at the Trx2 locus.
(a) Genomic organization of the Trx2 locus. The integration site for the knock out targeting cassette is indicated by a red arrow. Region of interest is shaded grey. 1 Unit on the scale = 1kb.   (b) Close up of the region of interest with the indication of genomic restriction sites (italics).   (c) Not1 restriction fragment termed genomic "Fe13" subclone containing the endogenous promoter (indicated by the black arrow) and the first two exons of Trx2. X4 and BE580 are DNA probes used for southern blot analysis.   Targeting construct Fe13lacZneo (d) and Fe13lacZhygro (e) are shown with construct specific restriction sites (normal). Grey shaded areas indicate homology arms used for homologous integration of both targeting constructs into the genomic Trx2 locus
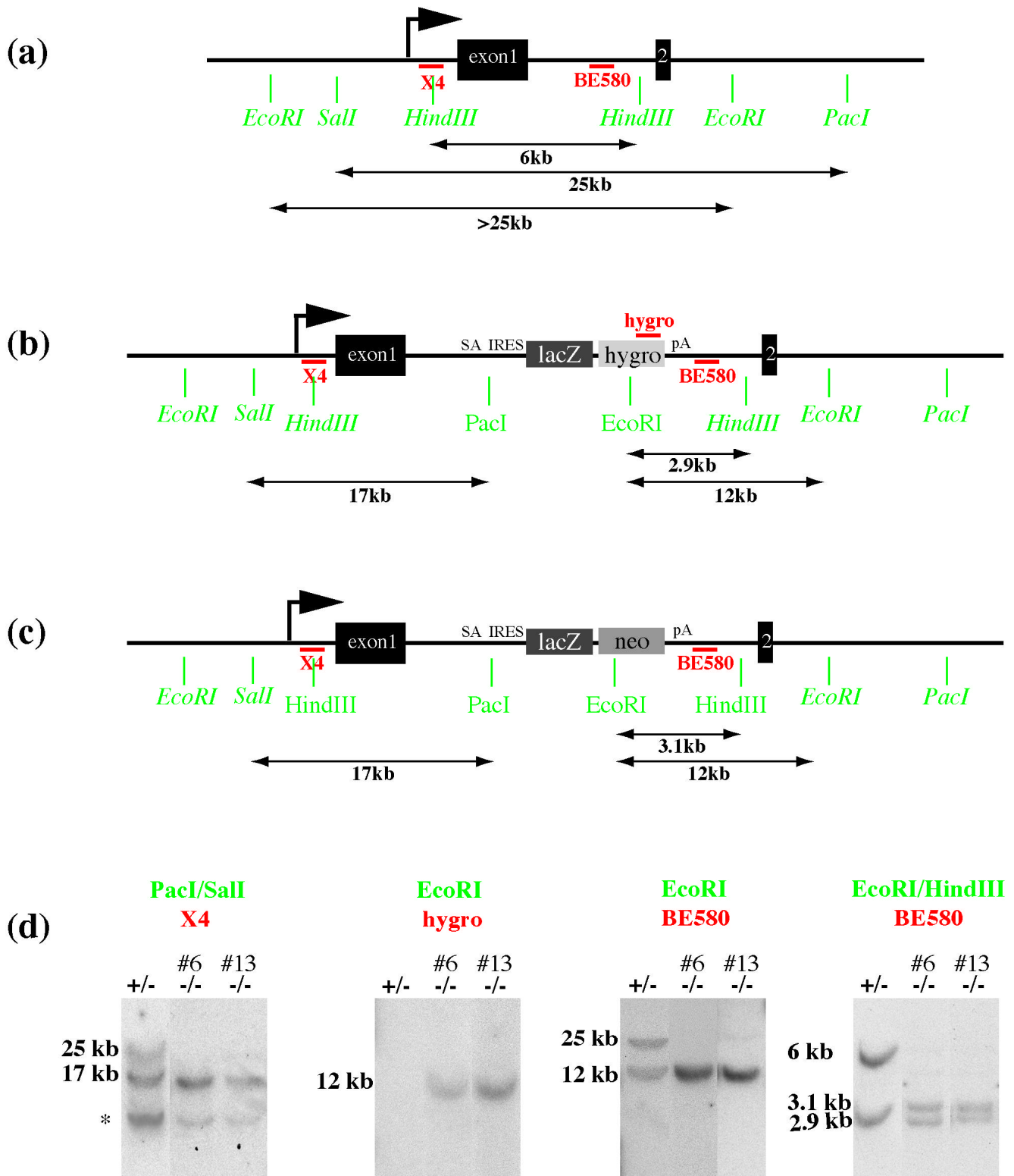
Figure. 5: **Southern blot strategy and analysis of Trx2 k.o. ES cells.**

Two Trx2 specific hybridization probes (X4, BE580) and a hygro specific hybridization probe (hygro) were used and are indicated in red at their hybridisation sites. Genomic restriction sites (italics) and target construct specific sites (normal) are shown in green with the resulting size of the digestion fragment indicated by a two-sided arrow below. **(a)** Wt allele **(b)** LacZ hygro targeted allele **(c)** LacZ-neo targeted allele **(d)** Southern digestion pattern of Heterozygous (+/-) Trx2 ES cells and two independent homozygous (-/-) clones #6 and #13 were digested with indicated enzymes (green) and hybrisized with hybridisation probes (red). Note that the +/- cells contain one lacZ-neo targeted allele (for detailed description please refer to text). Background bands are indicated by *.

addition to the 17kb-band corresponding to the targeted allele, the 25kb wt band is still visible in heterozygous cells while it is completely absent in doubly targeted ES cells. To further support the assumption that also the 3' side of the construct integrated correctly, fragment BE 580 (annealing 3' of the polyA signal) was probed onto an EcoRI digested blot and produced a 12 kb band representing the targeted allele, and a 25 kb band representing the wt allele which is only present in cells singly targeted with only the lacZ-neo construct (= heterozygous cells). In order to discriminate between the two different targeting cassettes (one carrying neo, the other hygro as selection marker), an enzyme combination digestion (EcoRI/HindIII) that differentially cuts in the neomycin and the hygromycin gene was selected and probed with BE580. The lacZ-neo allele creates a 2.9 kb band while presence of the lacZ-hygro allele is reflected by presence of a 3.1kb-band. The wt allele (6kb) was not detectable in both tested -/- cell clones, but both targeted bands were visible. This indicated that targeting was terminated successfully and that presence of both k.o. cassettes and absence of a wt allele was confirmed.

Southern analysis of DNA extracts from 96 stable transformants revealed a rate of 3% of homologous integrations. Three clones out of the analyzed 96 were homozygous for the Trx2 k.o. Two clones (#6 and #13) were thawed from the 48-well-dish, expanded and frozen for further experiments.

R-1.3 No Trx2 mRNA transcripts are detectable in k.o. ES cells

Even though integration of the k.o. cassette interrupts Trx2 transcription after exon1, downstream promoters or cryptic splice sites could lead to the production of a truncated version of the Trx2 gene that could retain some of the natural functions and obscure the analysis of Trx2 function in k.o. ES cells. To confirm the assumption that a complete knock-out of Trx2 had been created, Trx2-mRNA transcripts were assayed by Northern analysis. CF14 is a Trx2 specific probe and does not hybridize with the ortholog of Trx2, Mll, or any other trxG family member (figure 6a).

Total RNA was extracted from wt, Trx2 heterozygous and Trx2-k.o. ES cells. RNA concentration was determined by OD measurement at 260 nm. Equal amounts of RNA were loaded and separated by 1% agarose gel electrophoresis. The ethidiumbromid-
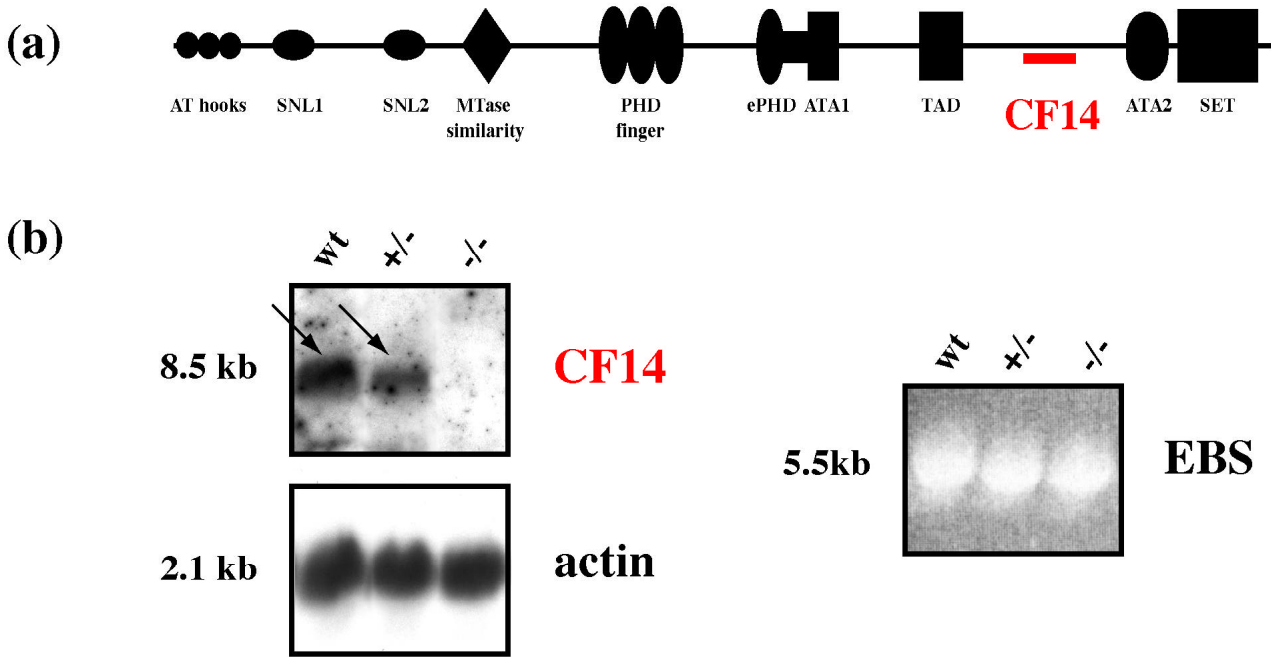
Figure 6: No Trx2 m-RNA trasncript is detected in Trx2 k.o. ES cells.
(a) Localization of the CF14 probe in a nonconserved region of the Trx2 transcript.   (b) Northern analysis of total RNA extracts from wt, Trx2 +/- and Trx2-/- ES cells. Probing with labelled Trx2 specific CF14 probe reveals that no Trx2 m-RNA is produced in -/- ES cells. The 8.5 kb Trx2 m-RNA fragment is indicated by black arrows. Probing the same blot with an actin probe and ethidium bromide staining of the 28s RNA (EBS) served as internal loading controls.
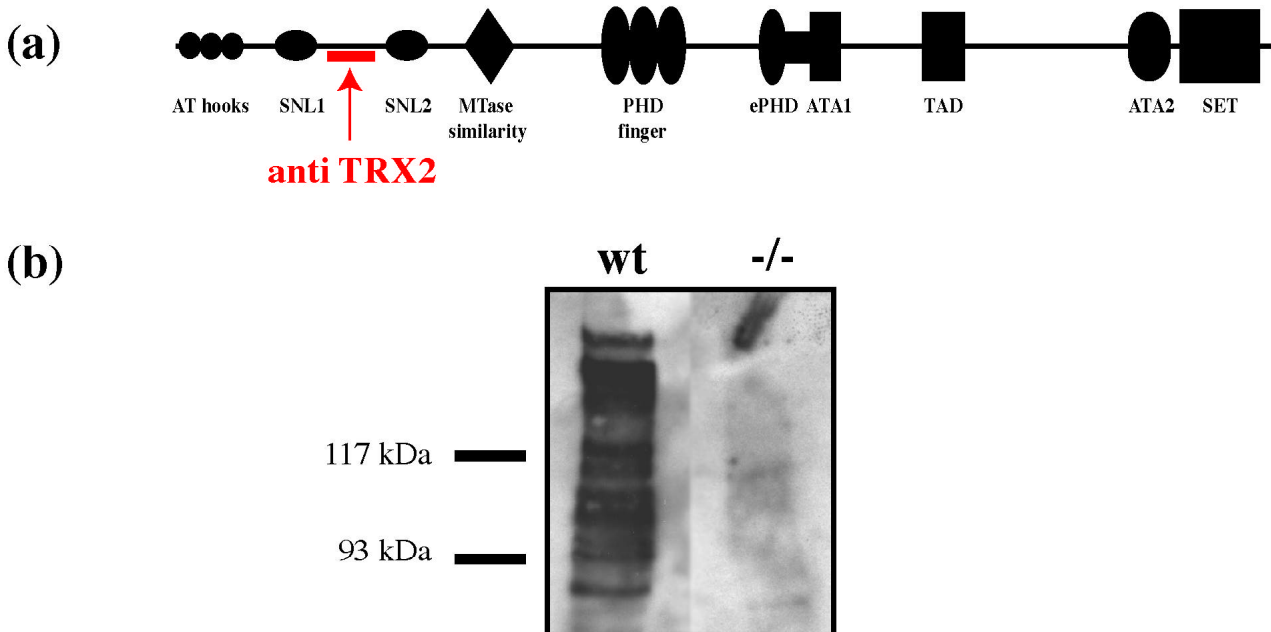
Figure 7: No Trx2 protein is detected in Trx2 k.o. ES cells.
(a) Localization of the TRX2 antibody in a nonconserved region of the Trx2 protein.   (b) Western analysis of wt and Trx2 -/- ES cell crude protein extracts. Probing with anti-TRX2 reveals that the 280 kD TRX2 signal and degradation products are only detected in wt ES cells but absent from Trx2 -/- ES cells. Coomassie staining of the gel confirmed that same amounts of total protein were loaded (data not shown). Molecular weight markers in kD are indicated left of the membrane.

stained gel showed non-degraded ribosomal 28sRNA of equal amounts. An actin probe served as an internal control that also confirmed RNA concentrations previously measured in the spectrometer.

Probing with the Trx2 specific probe CF14 visualizes the 8.5 kb wt Trx2 RNA fragment present in wt and heterozygous ES cells. Loss of one Trx2 allele (in +/-) results in an about 50% reduction of the signal while no Trx2 transcript is detectable in Trx2 -/- ES cells (figure 6).

Thus the integration of the k.o. cassette interrupts the entire production of full length and truncated Trx2 transcripts.

R-1.4 No TRX2 protein is detectable in k.o. ES cells

The need for a proper immunodetection tool lead to the design and recombinant expression of a TRX2 specific peptide for antibody production (see method section M-24). Eight earlier attempts to recombinantly express a section of TRX2 failed due to toxicity in *E. coli.* Only one TRX2 specific segment could be expressed and purified in satisfying amounts. This antigen was injected into rabbits for antiserum production. Affinity purification of the apparently lowly specific serum provided the TRX2 specific polyclonal antibody anti-TRX2.

Crude protein extracts from ten confluent 15-cm-dishes of wt or Trx2-k.o. ES cells were prepared and separated on a 5% polyacrylamid gel. After protein transfer the nitrocellulose membrane was probed with anti-TRX2. As secondary antibody an alkaline phosphatase conjugated anti-rabbit antibody was used for chemiluminescent detection (figure 7). No signal in the trx2 k.o. extracts was detectable. Therefore it was concluded that no TRX2 protein is translated in ES cells homozygous for the knock-out cassettes.

Northern and western analysis confirmed that indeed the k.o. strategy proved to be successful. No Trx2 transcript is produced and Trx2 -/- ES cells therefore represent a potent model to investigate Trx2 function in vivo.

R-1.5 Basic properties of Trx2 -/- ES cells

The ability to obtain and expand Trx2-/- ES cells already delivers the conclusion that these cells are able to survive without any functional Trx2 allele. To examine this in further detail, we performed several experiments comparing cell cycle distribution, proliferation rate, rate of apoptosis and colony formation properties between wt ES cells, Trx2 heterozygous ES cells and ES cells homozygous for the Trx2 k.o. Since during the course of creation Trx2-/- cells underwent two rounds of targeting and were exposed to double antibiotic selection during all cell passages, a Trx2+/- clone that had been processed in exactly the same targeting and selection procedures, but randomly rather than homologously integrated the second targeting cassette, was taken as a control.

R-1.5.1   Trx2 -/- ES cells display a wt distribution within the cell cycle

The most prominent approach to determine the cell cycle stage is based on measurement of cellular DNA content. This allows discrimination between cells in $G_{0/1}$ versus S versus G_2/M phases. DNA is generally stained with a fluorescent dye and cellular fluorescence is measured by flow cytometry (Crissman and Steinkamp, 1993; Current protocols, Interscience). The intensity of fluorescence integrated over the analyzed cell is in stochiometric relationship to the DNA content and therefore used to determine the cell cycle stage. Propidium iodine (PI) stains DNA via intercalation and emits at wavelengths above 610 nm. Discrimination of cells in particular phases of the cell cycle on the basis of differences in DNA content gives an insight into the cell's ability to perform basic vital functions.

Cells from comparable passage numbers were held in culture for five days (= 2 passages) before starting the experiment. Figure 8 shows data table and graphical display of the experiment. The graphs assign the number of cells counted over one specific PI fluorescence intensity. Neither Trx2 +/- clone #83, nor Trx2 -/- clone #13 show any significant difference in cell cycle distribution compared to wt ES cells.
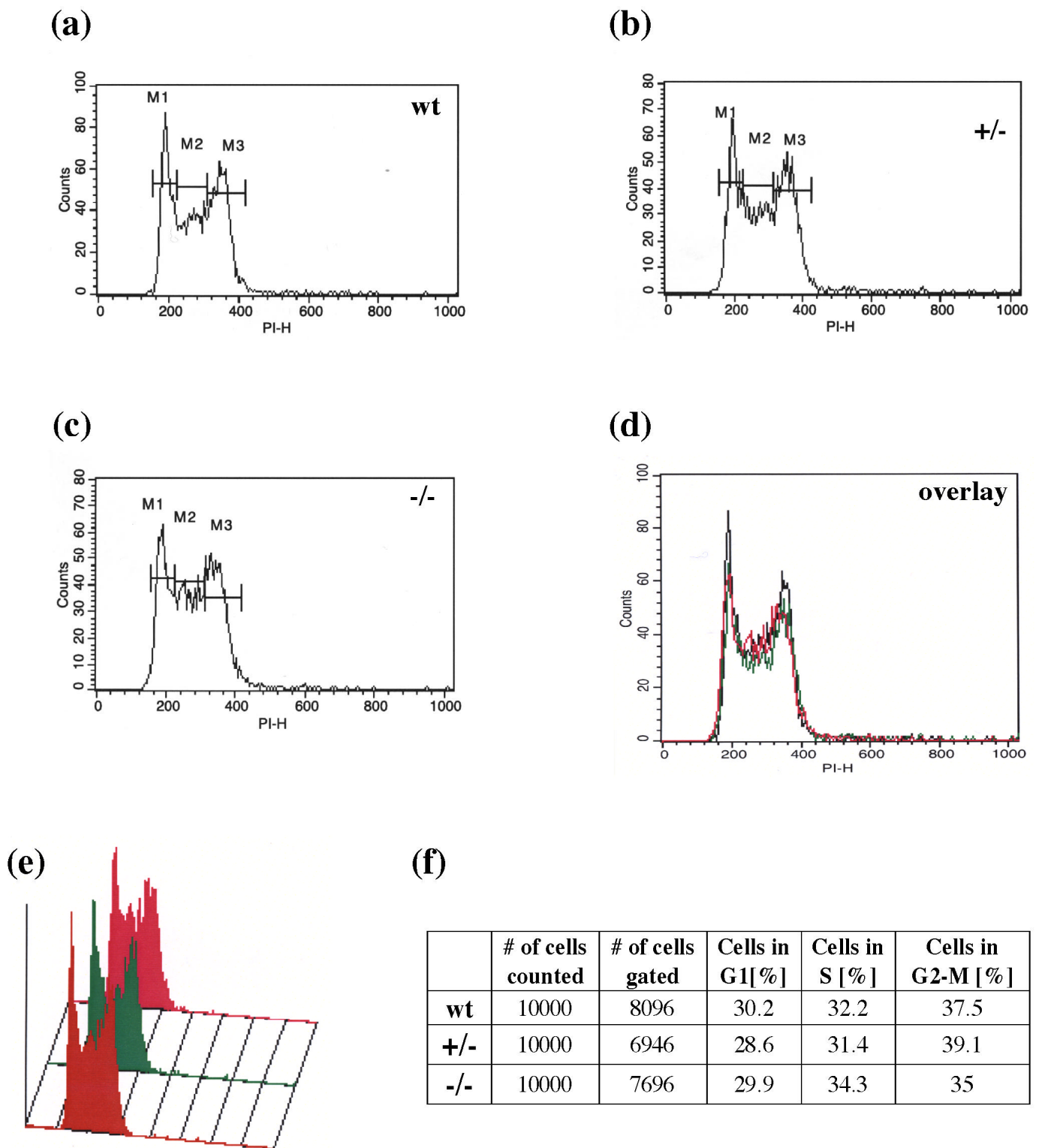
95

(a)

(b)

(c)

(d)

(e)

(f)

	# of cells counted	# of cells gated	Cells in G1 [%]	Cells in S [%]	Cells in G2-M [%]
wt	10000	8096	30.2	32.2	37.5
+/-	10000	6946	28.6	31.4	39.1
-/-	10000	7696	29.9	34.3	35

Figure 8: Trx2-/- ES cells display a wt distribution within the cell cycle.
Wt, Trx2 +/- and -/- ES cells were PI stained and sorted by FACS analysis.   **(a)** Graphical display of Trx2wt ES cell distribution in the cell cycle.   **(b)** Graphical display of Trx+/-t ES cell distribution in the cell cycle   **(c)** Graphical display of Trx2-/- ES cell distribution in the cell cycle.   **(d)** and **(e)** Overlay of wt (red) +/- (green) and -/- (pink). X-axis: PI fluorescence intensity in arbitrarily chosen units; y-axis: number of cells counted.   **(f)** Table of FACS data. 10000 cells per cell line were counted. Living cells were gated and seperated into M1, M2 and M3 that indicates progression through cell cycle according to DNA content. M1= cells in G1or G0; M2= cells in S-phase; M3= G2-M. The same gating parameters were used for all three cell-lines.

R-1.5.2 **Loss of TRX2 protein in k.o. ES cells does not cause a proliferation defect.**

Comparison of cell numbers reached after a certain incubation time provides information about the proliferative potential of the cell line. The expected average generation time for ES cells is approximately 18-24 h.

In the following proliferation assay wt, Trx2+/- and Trx2-/- ES cells from comparable passage numbers were plated in triplicates on either gelatin-coated or onto feeder-containing plates, trypsinized and counted after 1-5 days. The raw data values in table 1a and 1b were projected into the graphical display in figure 9 and figure 10 respectively..

For the first experimental approach termed "count-experiment" cells were plated, and one sample each was counted and discarded every 24 hours for five consecutive days (figure 9). For the second experimental approach termed "split-and-count-experiment", cells that had been counted on day one, were reseeded onto gelatin-coated or feeder-containing plates respectively. The same cell sample was again counted after 24 hours, again replated and so on. Trypsinization and reseeding was also performed on five consecutive days (figure 10).

As a general observation one could conclude that all cells growing on feeders (figure 9a and 10a) were proliferating faster than cells growing on gelatin-coated-dishes (figure 9b and 10b). Comparison of the three different cell clones though revealed no difference in proliferation behavior. All cells proliferated in the expected exponential growth curve.

In the "split-and-reseed-experiment" a general reduction in proliferation rate could be observed. This reduction can be explained and should be expected simply because the cells are stressed much more when trypsinized every 24 hours. Aside from these increased stress conditions, double targeted cells both +/- and -/- additionally display weaker overall condition caused by higher passage numbers and intense selection procedures. Therefore both clones have an even lower proliferation rate than wt cells in the "split-and-reseed-experiment". However, no significant difference between +/- and Trx2 k.o.-cells was detectable. Thus no proliferation phenotype caused by the absence of a functional Trx2 allele in Trx2-/- ES cells could be concluded.

	day 1		day 2		day 3		day 4		day 5	
	G	**FH**	**G**	**FH**	**G**	**FH**	**G**	**FH**	**G**	**FH**
wt	4	8.4	9.6	17.6	22.2	33	21	48	82.2	92.4
	3.5	7.2	9.1	15.4	26.1	36.8	31	63	43.8	96
	4.2	6.3	9.1	16.8	14	34.8	37	68	57.6	114.6
average	3.9	7.3	9.3	16.6	20.7	34.9	30	60	60.4	101
+/- ds	38	6.7	7.6	9.9	17	23.8	24	41.5	69.6	106.2
	24.5	5.2	6.4	9.9	15.6	25.4	37.5	56.5	42.6	88.2
	31.4	6.3	9.1	12.1	21	25.9	25.6	56	42	88.2
average	3.1	6.1	7.7	10.6	17.9	25	29	51.3	51.4	94.2
-/-	30	8.0	7.3	12	18	28.6	24.5	66	40.2	106.2
	22.3	6.0	6.6	17.5	18.8	32.6	28.5	67.5	46.8	81.8
	34.7	6.8	9.3	14.6	17.6	28.8	35	64	57.6	97.8
average	2.9	6.9	7.7	14.7	18.1	30	29.3	65.8	48.2	95.2

Table 1a: Raw data table for proliferation assay "Count-experiment".
Comparing counts of Trx2 wt, +/- and -/- cells cultured on feeder containing (FH) or on gelatine coated (G) plates. Cells were plated once in triplicates and counted every 24 hours for 5 consecutive days. The raw data table shows the single value for each samle and the average value from the triplicates (numbers x 10^5).

|	day 1		day 2		day 3		day 4		day 5	
	G	FH	G	FH	G	FH	G	FH	G	FH
wt	4	8.4	8.9	19.8	15.8	32.4	25.7	42.4		
	3.5	7.2	8	12.3	16.8	25.8	34.6	50	67.2	65.4
	4.2	6.3	9.2	13.3	14.2	23.8	29.7	63.3	65.2	74.4
average	3.9	7.3	8.7	15.1	15.3	27.3	30	51.9	66.3	69.9
+/- ds	3.8	6.7	5.5	9	7.4	14	16.8	31.2	30	42.6
	2.5	5.2	5	8.9	9.6	14.1	25.2	30	28.2	42.6
	3.1	6.3	5.2	10.3	7.3	13.9	16.2	38.4		
average	3.1	6.1	5.2	9.4	8.1	14	19.4	33.2	29.1	42.6
-/-	3	8.0	4.7	11.9	5.9	21.6	11.4	36.6	18.6	40.2
	2.2	6.0	4.4	13.3	8.5	27.4	13.8	31.2	24	52.2
	3.5	6.8	5.2	12.9	9.3	24.9	16.2	31.2		
average	2.9	6.9	4.8	12.7	7.9	24.6	13.8	33	21.3	46.2

Table 1b: Raw data table for proliferation assay. "Split-and-count-experiment".
Comparing counts of Trx2 wt, +/- and -/- ES cells cultured on feeder containing (FH) or on gelatine coated (G) plates. Cells were plated in triplicates and counted after 24 hours. After counting, cells were replated for next day's counting. This exercise was performed for 5 consecutive days. The raw data table shows the single value for each samle and the average value from the triplicates (numbers x 10^5).
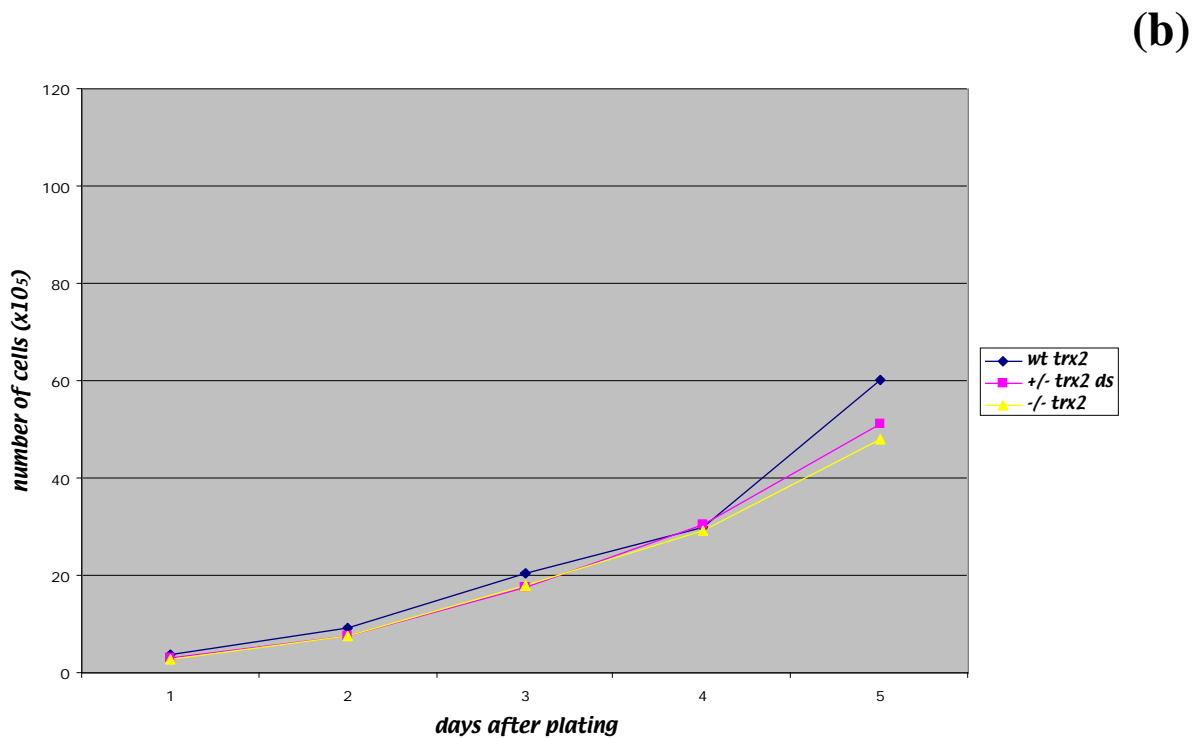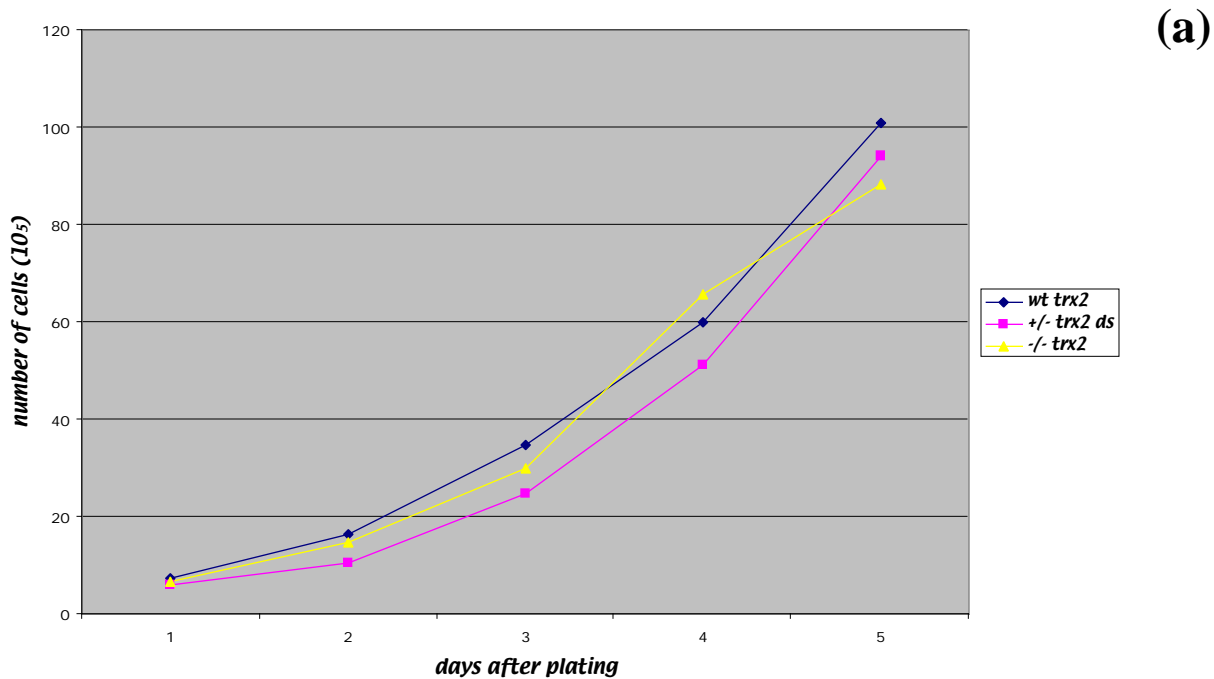
Figure 9:   Loss of TRX2 protein in k.o. ES cells does not cause a proliferation defect.
Graphical display of the data table 1a.   (a) average values for cells plated on feeders   (b) average values for cells plated on gelatine coated dishes. Counts for wt (blue), +/- (pink) and -/- (yellow) cells over 5 days.
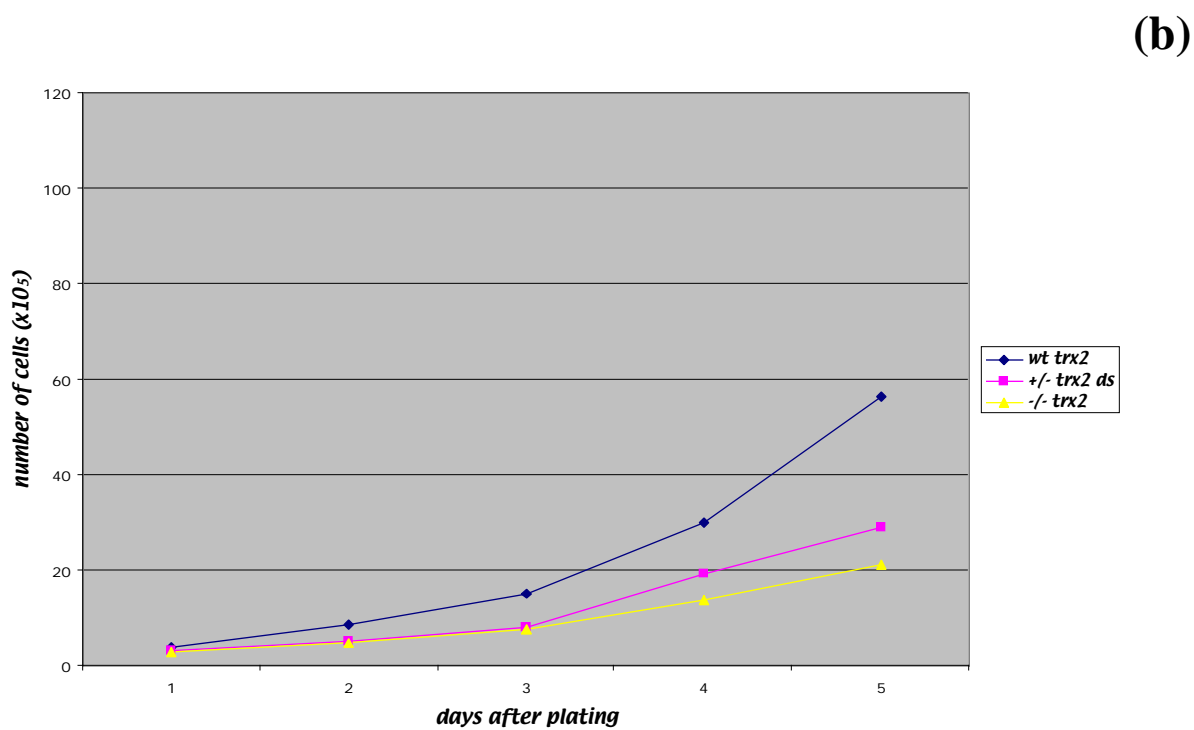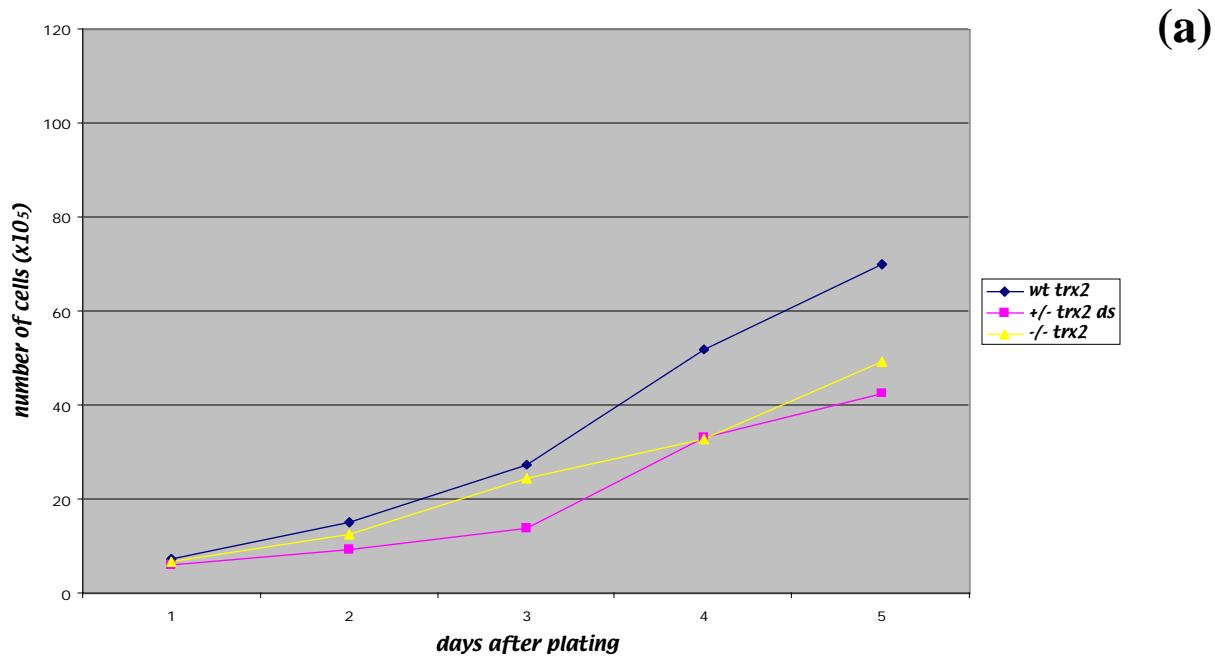
Figure 10:   Loss of TRX2 protein in k.o. ES cells does not cause a proliferation defect.
Graphical display of the data table 1b.   (a) average values for cells plated on feeders   (b) average values for cells plated on gelatine coated dishes. Counts for wt (blue), +/- (pink) and -/- (yellow) cells over 5 days.

R-1.5.3   Trx2 -/- ES cells display no colony formation defect

Appearance and size of colonies and also numbers of colonies formed by ES cells can give insights into their differentiation status, proliferation abilities and survival rates (Joyner, 2000). These basic properties were investigated with respect to the loss of TRX2 protein.

Equal amounts of wt, Trx2+/- and Trx2-/- ES cells were plated in quadruplicates onto feeder-containing 6-well-plates and grown for eight days with occasional media change. For better documentation formed colonies were fixed and stained with methylene blue, a dye that intercalates into DNA. The comparison of methylene blue stained colonies is shown in figure 11. All tested cell types show the same colony morphology (figure 11b) and also average colony numbers of about 1200 per well were seen for all cells (figure 11c). It was concluded that Trx2 -/- cells sustain the ability to form colony morphology undistinguishable from wt colonies.

R-1.5.4   Trx2 -/- ES cells display no enhanced rate of apoptosis

Initiation of the apoptotic program in a cell prohibits the propagation of malformed or misregulated cells (Wyllie et al., 1980). An increased apoptosis rate in exponentially growing ES cells is therefore an indication for loss of a vital function or disturbance of the cell cycle. To investigate if TRX2 is involved in any of those processes, we tested -/- cells for increased apoptotic cell death.

To compare apoptosis in wt and -/- ES cells, the TUNEL assay as available in the *In Situ* Cell Death Detection Kit (Roche) was used. The principle of the Kit is based on the fact that cleavage of genomic DNA during apoptosis results in strand breaks ("nicks"). Those DNA strand breaks can be identified by labeling free 3'OH termini with modified nucleotides in an enzymatic reaction. The Kit performs labeling of DNA strand breaks, by terminal deoxynucleotidyltransferase, which catalyzes polymerization of fluorescein-labeled nucleotides to free 3'-OH DNA. Incorporated fluorescein is detected by the anti-fluorescein antibody Fab and can be taken as a measure for the rate of apoptosis.

wt   **+/-**   **-/-**

(a)

(b)

(c)   **1235 colonies**   **1294 colonies**   **1140 colonies**
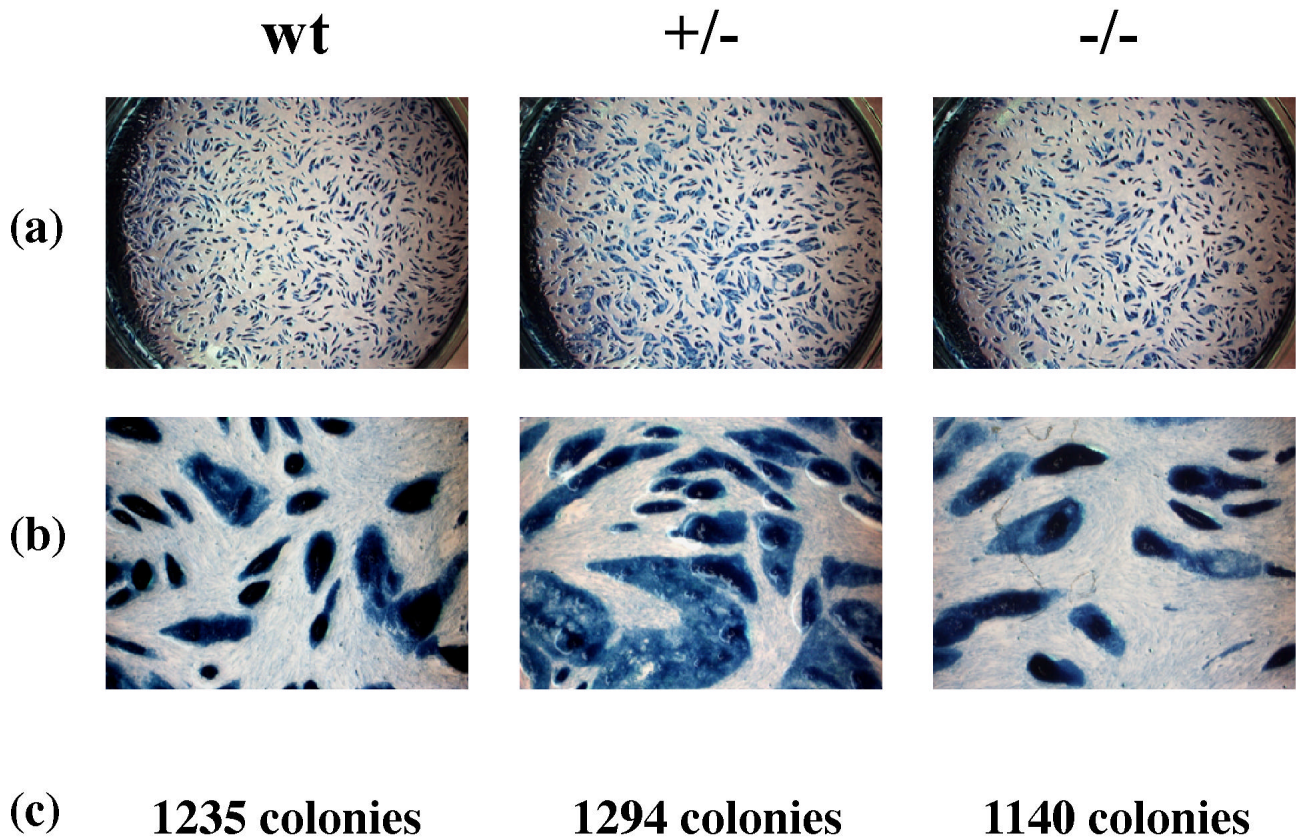
Figure 11: Trx2 -/- ES cells display no colony formation defect.
ES cells from wt (left), Trx2+/- (middle) and Trx2-/- (right) lines were cultured on gelatine-coated 6-well tissue culture plates and stained with methylene blue after 8 days. **(a)** The overview shows no difference in local distribution. **(b)** The close up shows no difference in morphological appearance. **(c)** The average total number of all colonies counted in the well do not indicate a significant difference.

Equal numbers of wt, Trx2+/- and Trx2-/- ES cells were plated onto either gelatin-coated or feeder-containing dishes and grown for five days with occasional media change. After application of the *In Situ* Cell Death Detection Kit, incorporation of fluorescein-labeled nucleotides equivalent to the rate of apoptosis was visualized under the fluorescence microscope.

A cell sample in which DNA strand breaks were induced by DNAse digestion served as a positive control, while a cell sample untreated with the reaction mix of the Kit served as a negative control. Both controls gave the expected signals as documented in figure 12a. In the positive sample small light dots all over the cell nucleus are evident. In the untreated negative control cells those signals were completely absent. Gelatin- and feeder-cultured cells behaved similar.

Figure 12b shows the results for the three different cell clones. In cells cultured on feeders no apoptosis could be observed. Gelatine-grown cells of all analyzed cell types show an increased number of apoptotic cells, but again no difference between wt, heterozygous and k.o. cells for Trx2 could be observed.

Therefore the absence of TRX2 protein in ES cells does not cause implementation of the apoptotic program.

All experiments concerning the basic properties suggest that Trx2-/- ES cells perform all vital functions and are therefore not distinguishable from wt ES cells by these assays.

At that point we wished to analyze Trx2-/- ES cells for their invitro differentiation potential, but were confrontet with the problem that the 129 E14 ES cell line used for targeting has no ability to differentiate in culture, but is instead strongly feeder and LIF dependent. The differentiation protocols applied caused massive cells death so that in vitro differentiation of ES cells had to be stalled.

(a)

positive control   negative control

G

FH

(b)

wt   +/- Trx2   -/- Trx2

G

FH
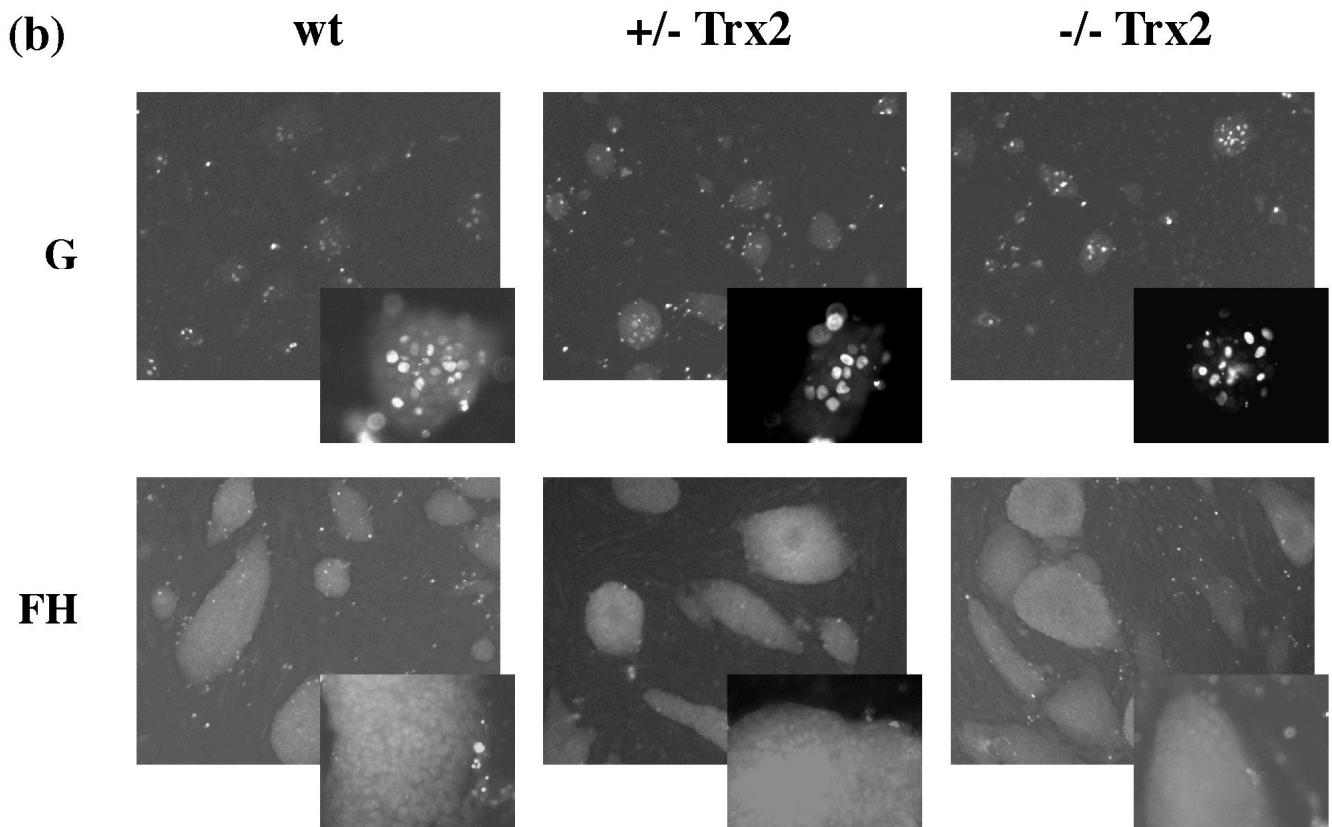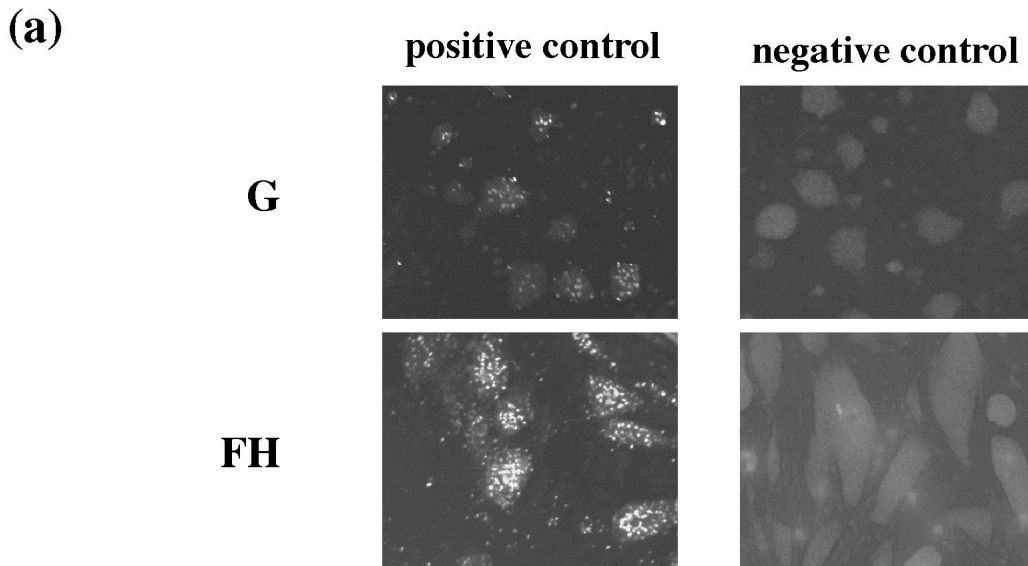
Figure 12:   Trx2 -/- ES cells display no enhanced rate of apoptosis.
Wt, Trx2 +/- and Trx2 -/- ES cells were cultured on feeder-containing (FH) or on gelatine-coated (G) cover slips as indicated.   **(a)** Controls. Positive control cells diplay increased apoptotic signals (induced by DNAse treatment); negative control cells (untreated with the labelling mix) do not display any unspecific background.   **(b)** Comparison of Trx2 wt, +/- and -/- cells shows the same rate of apoptosis for all cell lines on gelatine plated cells. Cells plated on feeder cells did not face apoptosis at all.

R-1.6 Blastocyst injections and analysis of chimeric embryos

In order to obtain insights into Trx2 function during mouse embryonic development, we decided to use Trx2 k.o. cells in blastocyst injection experiments. Injection of genetically modified ES cells into the inner cell mass of wt blastocysts leads to random integration of the transgenic ES cells into the embryo proper and progenitors of these cells can locate anywhere in the chimeric embryo (Bradley et al., 1984). Statistical exclusion of injected k.o. ES cells from specific regions of the embryo might indicate the importance of the functional allele in these particular regions. Analysis of the distribution of Trx2 -/- cells within Trx2-/- chimeric embryos could reveal important requirements for TRX2 during embryonic development.

Blastocysts from mice were isolated at embryonic day E4.5. After injection of 20-25 transgenic ES cells per blastocyst, 14 blastocysts were reintroduced into the uterus of pseudopregnant foster mothers (seven into the left and seven into the right uterus arm). Developing embryos were extracted for analysis at various developmental stages. As a control Trx2 heterozygous cells (doubly targeted and selected) were injected in parallel. The Trx2 heterozygous mutation neither has embryonic nor adult phenotypes (our unpublished results); an overall contribution of +/- cells was therefore expected.

To monitor the distribution of Trx2 -/- and heterozygous cells we used the feature of the k.o. targeting construct to expresses the lacZ gene under the endogenous Trx2 promoter. Chimeric embryos were lacZ stained either as whole mount or in sections according to the size of the embryo at the corresponding developmental stage. Finding of blue-stained cells within the chimera indicates contribution of cells that express lacZ and therefore carry one (in Trx2 heterozygous ES cells) or two (in homozygous Trx2 k.o. ES cells) targeted Trx2 alleles. Analysis of the chimeric embryos was focused on the identification of organs, tissues or cell types in which Trx2 -/- cells did not participate.

Table 2 shows a summary of all analyzed embryonic stages and lists the analysis procedure. Two litters each were analyzed for embryonic stages E8.5 and E9.5 and four litters each to analyze embryonic stages E10.5 and E18.5. While for E8.5, E9.5 and E10.5 whole mount stainings were still possible, size limitations for the E18.5 embryos required

stage	# pups	lacZ whole mount staining				TOTAL (whole mount)				lacZ kryosection staining				TOTAL (kryosection)			
		# analysed	high	low	not	# analysed	high	low	not	# analysed	high	low	not	# analysed	high	low	not
E8.5	9	9	1	4	4	18	2 11%	10 56%	6 33%								
	9	9	1	6	2												
E9.5	9	9	2	5	2	18	2 11%	10 56%	6 33%								
	9	9	/	5	2												
E10.5	8	8	1	6	1	28	2 7%	11 39%	15 54%								
	5	5	/	2	3												
	6	6	/	3	3												
	9	9	1	/	8												
E19.5	9									4	/	3	1	17	/	14 82%	3 18%
	4									4	/	3	1				
	12									4	/	4	/				
	14									5	/	4	1				

Table 2:   Analysis of chimeric embryos resulting from Trx2 -/- ES cell injections into blastocysts.

staining of kryosections. The pups from every litter could be separated into 3 different categories according to the contribution of blue stained -/- cells to the chimeric embryo:

1. Non chimeric embryos (n.c.) were completely white with no contribution of Trx2 -/- blue lacZ-stained cells.
2. Low content chimeras, displayed a minor (up to 50%) contribution of Trx2 -/- blue lacZ-stained cells.
3. High content chimeras displayed a higher contribution (>50%) of Trx2 -/- blue lacZ-stained cells.

Injection of control Trx2 heterozygous cells as expected resulted in a homogenous blue staining all over the embryo, which has a wt appearance (figure 13 and 14 middle panel, figure 15). That means Trx2 +/- cells contribute to every tissue of the developing embryo at all stages without compromising vital cell functions. It also shows that the injection procedure was performed successfully, that injected ES cells kept their pluripotency and were therefore able to integrate into the inner cell mass of the host blastocyst. Since the blue staining reflects endogenous Trx2 expression, this control injection additionally confirmed previous observations of ubiquitous expression obtained with Trx2-RNA in situ hybridisations (our unpublished results). TRX2 protein is continuously and ubiquitously expressed through all analyzed embryonal and fetal stages.

Non-chimeric embryos looked like wt embryos and were neglected in further analysis.

In the **low chimeric embryos** (figure 13 and 14 right panel) development proceeds normally. These embryos were examined for Trx2 -/- less tissues (figure 13). At E8.5, E9.5 and E10.5 blue staining appears spotted all over the embryo. To evaluate the impression that stainings in posterior (tail) regions were more prominent than in anterior (brain) regions and to investigate if blue stainings in all structures were not only superficial, kryosections of the already stained embryos were performed. However in these 50µm embryonic sections, the contribution of blue-/- cells could not be excluded from any tissue.
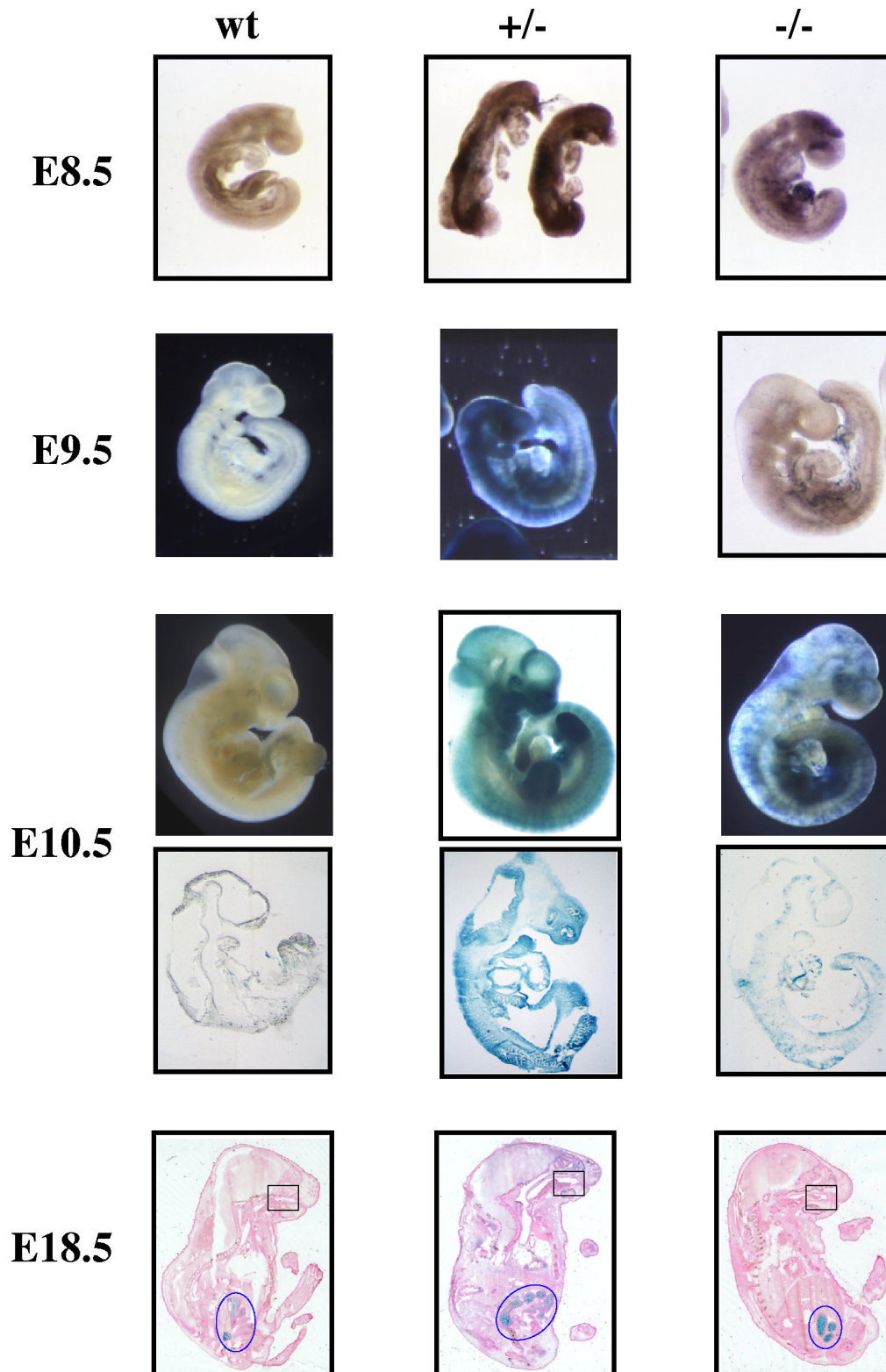
Figure 13: **Trx2 k.o cells are eliminated from the chimeric embryo until E18.5**.
LacZ staining of Trx2 wt , +/- and low chimeric Trx2 -/- embryos. Whole mount lacZ staining was performed for developemental stages E8.5, E9.5 and E10.5 (rows 1-3). For E10.5 additionally (row 4), for E18.5 exclusively (row 5) embryo kryosections were made. LacZ stainings of wt embryos are shown in the left column, stainings of chimeric embryos rerated by blastocyste injection of Trx2+/- ES cells are shown in the middle column and display a homogenous contribution of +/- cells to every tissue of the embryo. Stainings of chimeric embryos created by blastocyst injection of homozygous Trx2 k.o. ES cells are shown in the right column. Those chimeric embryos display a diverse distribution of blue -/- cells until E10.5. Elimination of blue cells from the embryos is evident at E18.5. Unspecific staining in the gut of E18.5 embryos (indicated by blue oval) is due to endogenous lacZ expression. A close-up of the area marked by a black square is shown in figure 14.
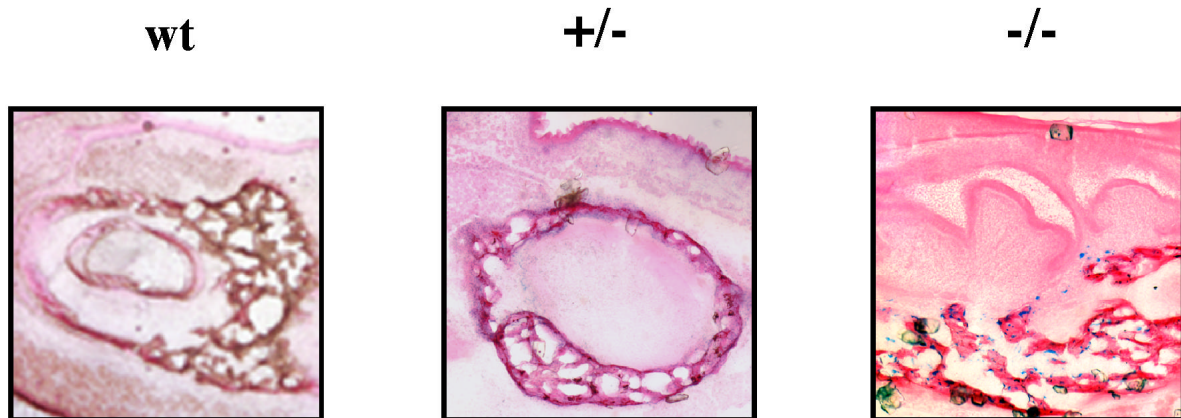
Figure 14: Trx2 -/- cells are only found in mandibular region of E18.5 chimeric embryos.
Mandibular region of kryosectioned embryos at E18.5. Close up of figure 13, in which Trx2 wt, +/- and low chimeric -/- embryos were dissected and kryosectioned at E18.5. Trx2 -/- cells can only be detected in osteoclasts and/or macrophages within the mandibular region, while Trx2+/- cells still homogenously contibute to that region.

From this it was concluded that Trx2 deficient cells still contribute to all tissues around E10.5. Next we were interested in the requirement for Trx2 in newborn pups. To exclude cannibalism of retarded litters, pups were examined one day before the natural birth (E18.5), kryosectioned and stained for lacZ expression: While dispersed blue staining cells were widely observed in heterozygous chimeras, almost no blue staining was apparent in Trx2 deficient cell chimeras (figure 13). Again we were not able to observe tissue specificity in the lack of -/- cells, rather widespread elimination throughout the embryo.

The last regions in which Trx2-/- lacZ-expressing cells could still be observed at E18.5 include macrophages and/or osteoclasts in cartilaginous areas forming bone. Figure 14 shows the mandibular region in the ventral part of the jaw bone (close from figure 13). In this ossification center clusters of lacZ expressing cells can be found. These seem to be the only cell types in which lack of Trx2 can still be tolerated at the end of embryonic and fetal development.

Once the proportion of k.o. cells was above a certain threshold (**highly chimeric embryos**), the embryos showed a retardation phenotype. In all analyzed stages (E8.5-10.5) the most affected embryos were around E8.5 (figure 15). After E10.5, high chimeric embryos were not found (data not shown). We assume high Trx2 -/- cell contribution causes a similar developmental retardation as the complete k.o. and that these embryos were resorbed before stage E18.5. The nature of retardation seen in highly chimeric embryos was very general. We observed an overall reduction in size and developmental advance without any regional specificity. This observation mirrors the phenotype of Trx2-/- embryos produced from Trx2 heterozygous crosses (our unpublished results). In both cases the analysis shows a general retardation in growth and development from E7.5 on.
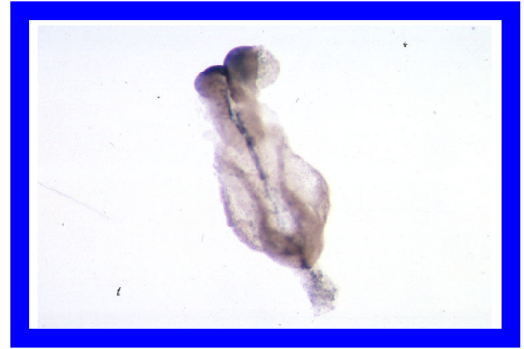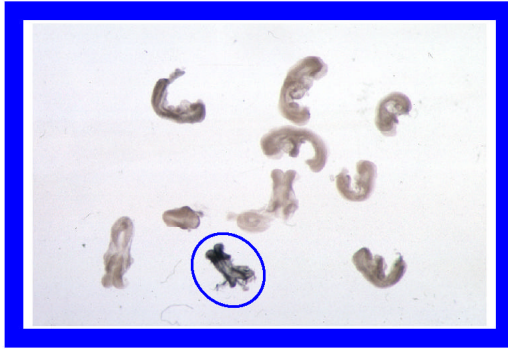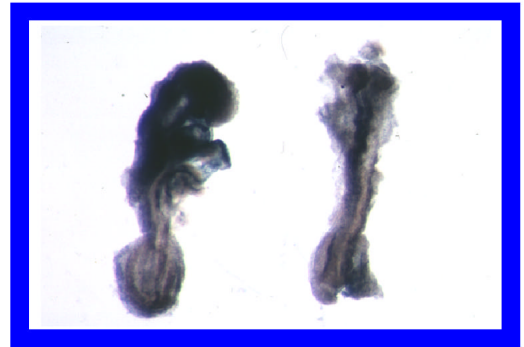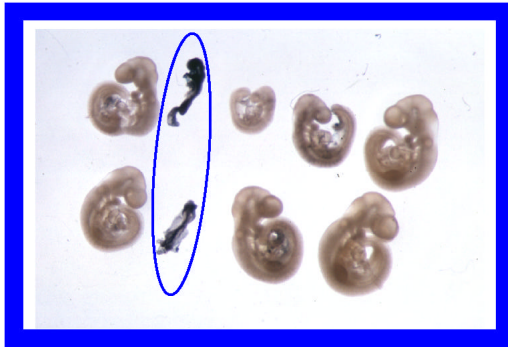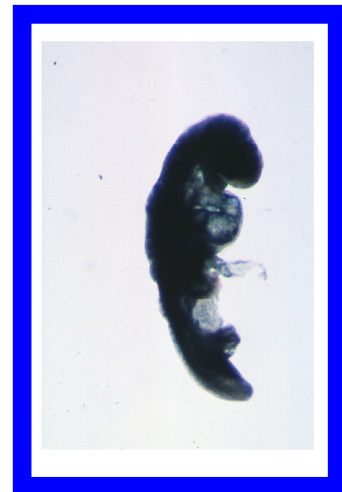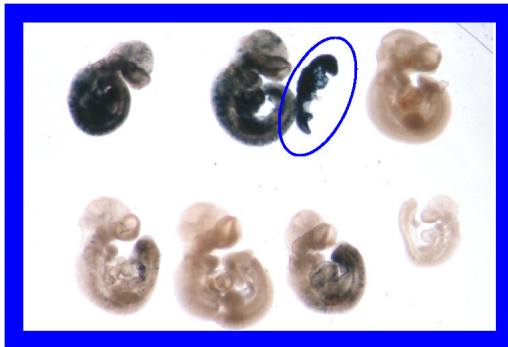
Figure 15:   Highly chimeric Trx2 -/- embryos display a retardation phenotype.
Litters created by blastocyst injections with Trx2 k.o ES cells were dissected at developmental stages E8.5, E9.5 and E10.5. Whole mount lacZ stainings of the litters were performed. As seen in the litter overview (left column) and in the close up of the blue circled highly chimeric embryos (right column), development of embryos with a high contribution of Trx2-/- cells in all analyzed developmental stages is retarded.

R-1.7 Histone methylation in TRX2-deficient ES cells

TRX2 is a candidate transcriptional regulator and contains a SET domain (FitzGerald et al., 1999). SET domains have been recently identified to convey histone methyltransferase activity to several proteins (for review see Lachner and Jenuwein, 2002). A very recent publication suggests that *Drosophila* TRX is associated with H3K4 methyltransferase activity (Czermin et al., 2002). Thus several experiments were performed addressing the potential role of TRX2 in histone methylation.

R-1.7.1   H3K4 methylation is unaffected in Trx2 deficient ES cells

To study the potential role of Trx2 in histone H3-K4 methylation, nuclei preparations from wt and Trx2 -/- ES cells were separated by 15% SDS-PAGE and transferred onto nitrocellulose membranes. An antibody specific against dimethylated H3-K4 was used to examine differences in the H3 K4 methylation pattern of the two ES cell lines.

Figure 16 shows that no difference between the wt and Trx2 -/- sample concerning the signal for H3 K4 methylation was observed. This result suggests that either Trx2 is not a H3 K4 methyltransferase, or that it is a minor H3-K4 methyltransferase in ES cells and/or that lack of Trx2 can be compensated by other methyltransferases in the cell.

R-1.7.2   Histone modification pattern is unaffaected in Trx2 -/- ES cells

To obtain a broader view of histone modifications in ES cells, we decided to separate nuclear extracts on Triton/Acid/Urea gels (TAU). This kind of separation allows resolution of post-translationally modified histone isoforms. Especially histones with varying levels of acetylation can be separated due to their charge differences. Since methyl groups are neutral with respect to electrical charge, unfortunately methylation states of histones cannot be separated due to lack of charge differences. But since there is an observed link between histone acetylation and methylation, changes in the methylation status could potentially be reflected by a differential acetylation pattern (Rea et al., 2000). Figure 17 shows the separation of nuclear extracts from wt and Trx2 -/- cells on a TAU gel. Commercially available (Roche) single histone (H1, H2A, H2B, H3 and H4) samples
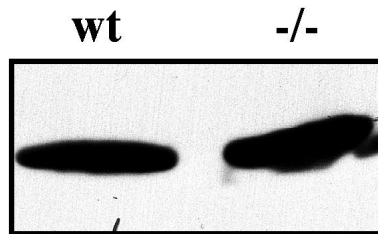
Figure 16: H3K4 methylation is unaffected in Trx2 deficient ES cells.
Comparison of H3K4 methylation in wt and Trx2 -/- ES cells. An antibody specific for H3 di-methylated at K4 detects same levels of H3K4 methylation in nuclear extracts from wt and Trx2 -/-ES cells.
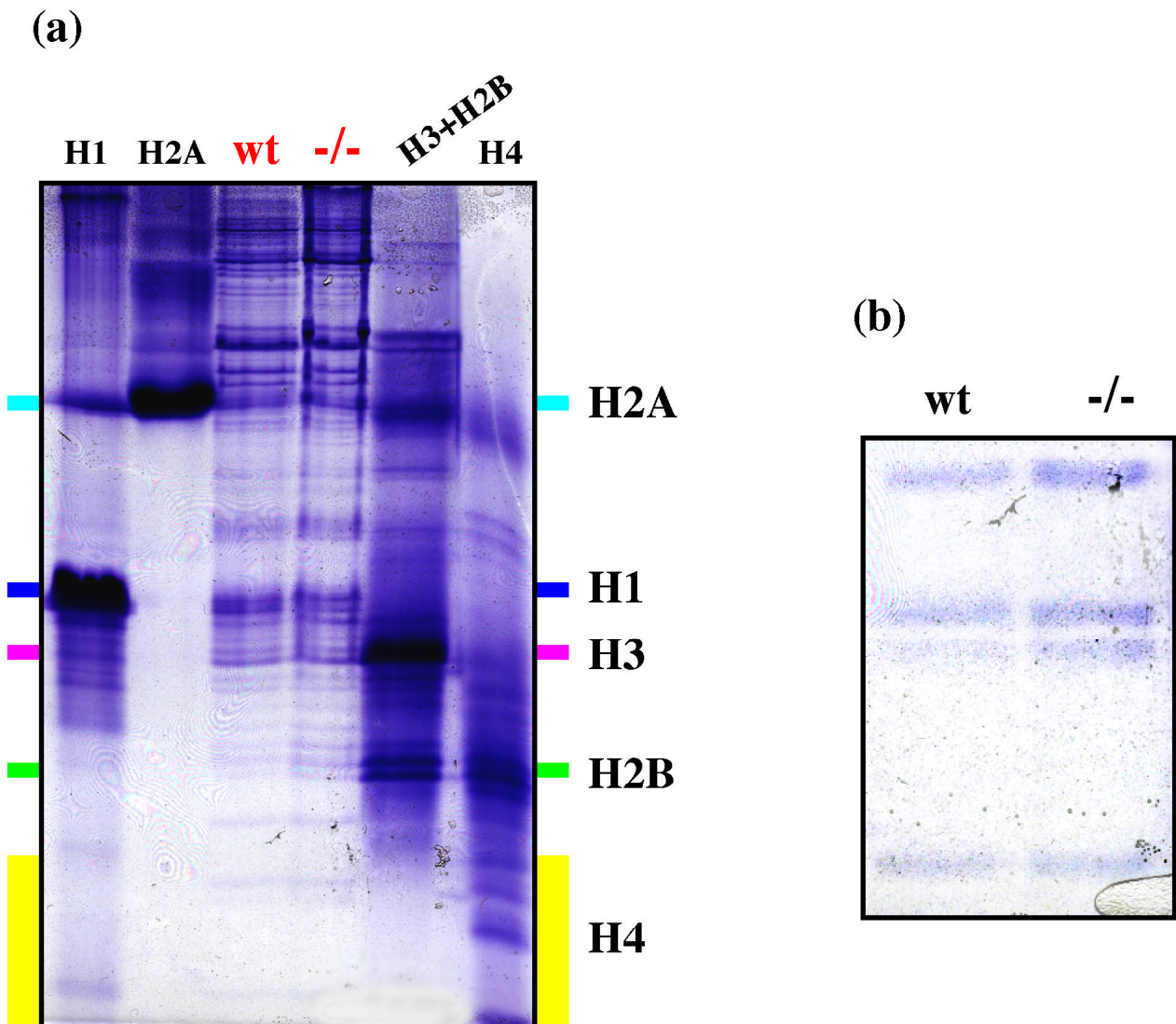

Figure 17: Histones of wt and Trx2 -/- cells display same pattern of posttranslational modifications.
Nuclear extracts of wt and Trx2 -/- ES cells were separated on a TAU gel. Proteins were visualized by Coomassie staining. Single histones were loaded as reference alongside. Colored bars mark the regions in the gel where isoforms of a particular histone should be expected (cyan: H2A; blue: H1; purple: H3; green: H2B; yellow: H4). Neither in the overview (a) nor in the close up (b) of the histone H4 region can a change in the pattern of posttranslational modifications be observed.

were run in parallel as controls; regions of the separated isoforms of each histone are indicated by colored areas.

Neither for histones nor for any other nuclear protein the posttranslational modification pattern was observably different. No differences between wt and Trx2 -/- samples concerning the acetylation status could be detected. It seems as if loss of TRX2 protein has no effect on the total posttranslational protein modification pattern in ES cells.

R-1.7.3 Histone methylation pattern is unaffaected in Trx2 -/- ES cells

In order to further investigate the involvement of TRX2 in histone methylation, wt and Trx2 -/- cells were incubated with the H^3-labeled methyl group donor S-adenosylmethionine (SAM). If histone methylation was mediated by TRX2 a difference between the wt and the -/- cells may be revealed by TAU gel electrophoresis and autoradiography. Histones can be labeled by either incorporating the radio-methyl-group of SAM into lysine, arginine or histidine residues of the histone tail or by incorporation of radiolabeled methionine into the peptide chain. Since only the former is a measure for the rate of tail methylation, it has to be discriminated from the latter event. To avoid incorporation of H^3 into the peptide backbone of proteins during protein synthesis, protein synthesis was inhibited by addition of cyclohexamide (10 µg/ml) in the culture media (Gravela et al., 1977; Puga et al., 2000). In the optimal case protein synthesis would be completely stalled and the autoradiograph of the TAU separation should only display histone tail methylation.

After 8 hours cells were harvested and nuclei were isolated. Nuclei samples were subjected to TAU separation, and incorporation of radio labeled methyl groups into histones was visualized by autoradiography (figure 18). As indicated by the high levels of background, protein synthesis had not been completely inhibited by cyclohexamide. By careful analysis still no difference in H^3 labeled histones was detected and it could be concluded that the overall methylation status of the Trx2-deficient ES cells was not altered.
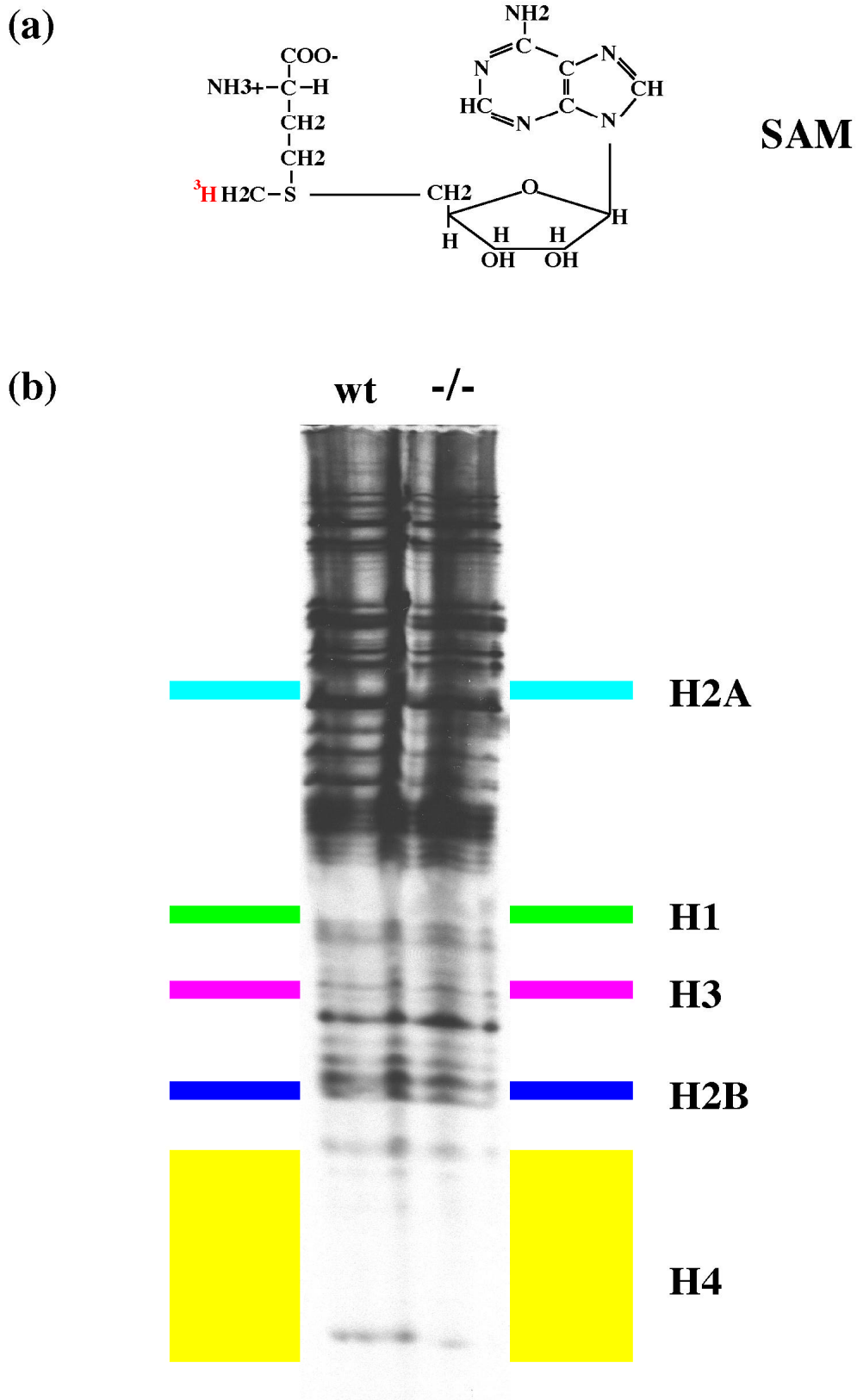
Figure 18: **Histones of wt and Trx2 -/- ES cells display same pattern of posttranslational methylation.**
(a) Chemical structure of the methylgroup donor S-adenosyl-methionine (SAM). The tritium labelled (3H) methyl group is indicated in red. (b) Autoradiograph. Wt and Trx2-/- ES cells were incubated with H3-S-Adenosylmethionine (SAM) in the presence of cyclohexamide and nuclei extracts were separated on a TAU gel. Background signal from non-histone proteins may be due to incorporation of labelled methionine during protein synthesis. As in figure 17, colored bars mark the histone regions in the gel (cyan: H2A; purple: H1; magenta: H3; green: H2B; yellow: H4). No difference between wt and Trx2 -/- ES cells concerning the methylation pattern can be observed.

R-2   *TRX2 fusion to EYFP (EYFP-TRX2)*

Green fluorescent protein (GFP) from jellyfish *Aequorea victoria* contains a fluorescent cyclic tripeptide (fluorophore) whose fluorescence is preserved in chimeric fusions with other proteins of interest (Chalfie et al, 1994). This fusion protein can be expressed in many organisms without disturbing the natural function and distribution of the original protein (Lippincott-Schwartz and Smith, 1997). It offers several advantages over conventional reporters such as lacZ or alkaline phosphatase, in that its visualization does not require a chromogenic substrate and can be realized in real time, in vivo and in situ. This opens numerous possibilities to study intracellular protein localization in the vital context, which is a worthy tool to investigate dynamic changes of protein distributions upon cellular processes. By directed mutagenesis of the fluorophore and interacting amino acids, firstly an enhanced GFP-version (EGFP) that gives a brighter fluorescent signal (Cormack et al., 1996; Yang et al., 1996), but also EGFP variants like ECFP (cyan), EYFP (yellow) and EBFP (blue) (Heim and Tsien, 1996; Yang et al., 1998; Miyawaki et al., 1997) differing in excitation and emission spectra have been created, so that now also double labeling and therefore co localization of intracellular proteins can be studied (Ellenberg et al., 1998; Ellenberg et al., 1999).

Since it is our particular interest to study the involvement of TRX2 in differentiation processes this in vivo imaging technique appeared to be the method of choice. Upon fusion of TRX2 to a fluorescent partner, we would be able to firstly learn about its cellular and sub cellular localization in undifferentiated ES cells but also to follow its distribution over the in vitro differentiation pathway or in terminally differentiated mouse tissue.

Our choice for the fluorescent fusion partner of TRX2 fell upon the enhanced yellow version of GFP (EYFP) since it can be combined with fusions to the enhanced cyan fluorescent protein (ECFP) to perform future co localization studies. EYFP has an excitation peak of 514 nm and emits at 527 nm, while ECFP is excited at wavelengths of 434 nm to emit light of 474 nm. Their brightness has been reported to allow detection of 8-10 co-localized FP molecules in a small cellular domain like the nucleus (Niswender et al., 1995).

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Publications about EYFP expressed in transgenic mice (Feng et al., 2000, Hadjantonakis et al., 2002; Metzger et al., 2002) and EYFP fusions to cellular proteins expressed in mammalian cell lines (Ellenberg et al., 1998) suggest that EYFP neither has a toxic effect in the mouse, nor influences the natural behavior of the fusion.
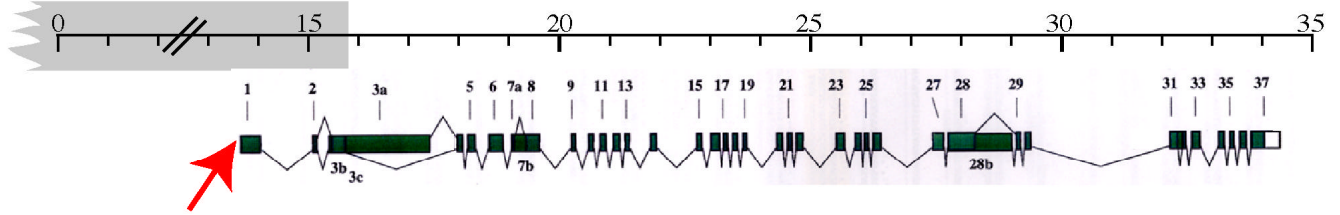
Both N-terminal and C-terminal fusions for EYFP have been described (Godwin et al., 1998, Kanda et al., 1998; Ellenberg et al., 1997; Presley et al., 1997, Drew et al., 2001; Baumann et al., 1998; Stradal et al., 2001). We choose to fuse EYFP to the N-terminus of TRX2 since the highly conserved SET domain reaches to the very C-terminus of the protein. EYFP in a C-terminal fusion was therefore feared to negatively influence the activity of the SET domain. The N-terminal AT-hooks, SNL and MT domains in contrast are located in a comfortable distance from the end of the TRX2 protein so that a functional disturbance of those domains was not expected by the fusion.

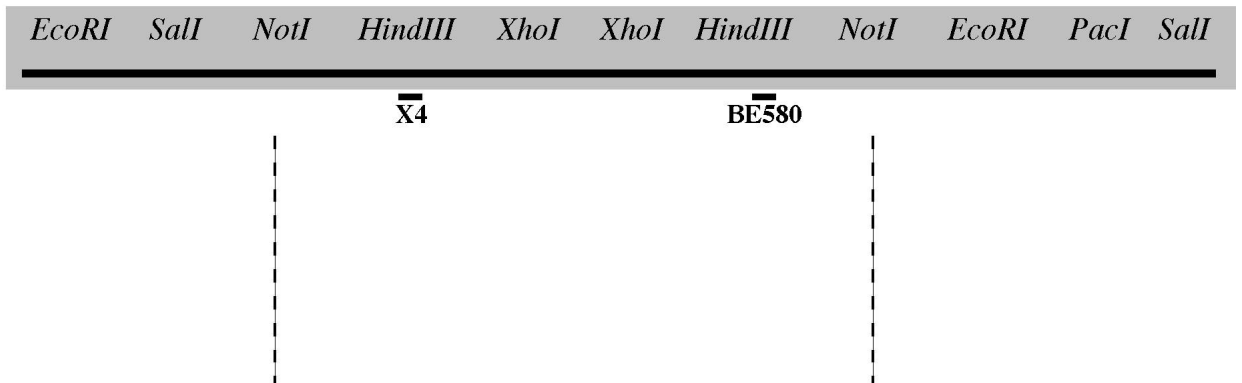## R-2.1 Creation of the EYFP-TRX2 fusion in ES cells

In order to study sub cellular localization of the endogenous TRX2 protein in vivo, an N-terminal knock-in to fuse EYFP to the endogenous Trx2 gene was created. Figure 19a displays the genomic organization of the endogenous Trx2 locus. The genomic sub clone Fe13 is a 16 kb NotI restriction fragment of the genomic region and contains the promoter region and the first two exons of Trx2 (figure 19b and c). This sub clone was modified in a way that the targeting cassette EYFP-neo (described in detail in the next paragraph) was cloned just behind the endogenous ATG of the Trx2 gene (figure 19d).

The targeting cassette displayed in figure 20e contains the EYFP coding region, and a neomycin selection marker flanked by loxP sites followed by a PolyA signal. Expression of neomycin is driven by both a prokaryotic (-lactamase promoter blaP) and a eukaryotic promoter (SV40 early enhancer/promoter region SVe), which allows selection for homologous recombination in both bacteria and ES cells. EYFP-neo is flaked by two homology regions XXX and OOO. Region XXX consists of 60 bp of the endogenous Trx2 promoter region including the starting ATG. Region OOO consists of the first 300 bp of the Trx2 exon one. These regions serve as homology arms to mediate ET/Red recombination of the targeting construct into Fe13.
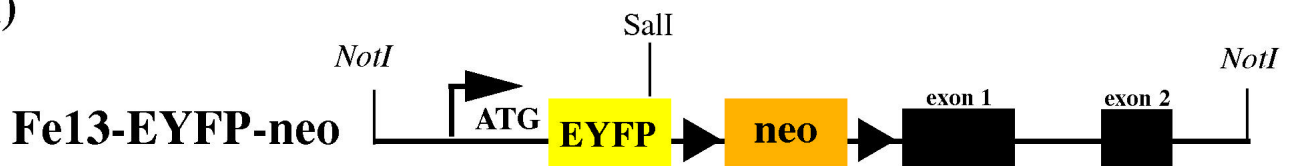
(a)

(b)

| EcoRI | SalI | NotI | HindIII | XhoI | XhoI | HindIII | NotI | EcoRI | PacI | SalI |

X4   BE580

(c)

Fe13

NotI   *NotI*

ATG   exon 1   exon 2

(d)

SalI

Fe13-EYFP-neo

NotI   *NotI*

ATG   EYFP   neo   exon 1   exon 2

Figure 19:   Genomic oragnization of the endogenous Trx2 locus.
(a) Genomic organization of the Trx2 locus. The integration site for the EYFP-fusion targeting cassette is indicated by a red arrow. Region of interest is shaded grey. 1 Unit on the scale = 1kb.   **(b)** Close up of the region of interest with the indication of genomic restriction sites (italics). X4 and BE580 are DNA probes used for southern blot analysis.   **(c)** Not1 restriction fragment termed genomic subclone Fe13 contains the endogenous Trx2 promoter and the first two exons.   **(d)** Targeting construct Fe13-EYFP-neo is shown with construct specific restriction sites (normal). The targeting cassette EYFP-neo is cloned just 3' of the endogenous Trx2-ATG.
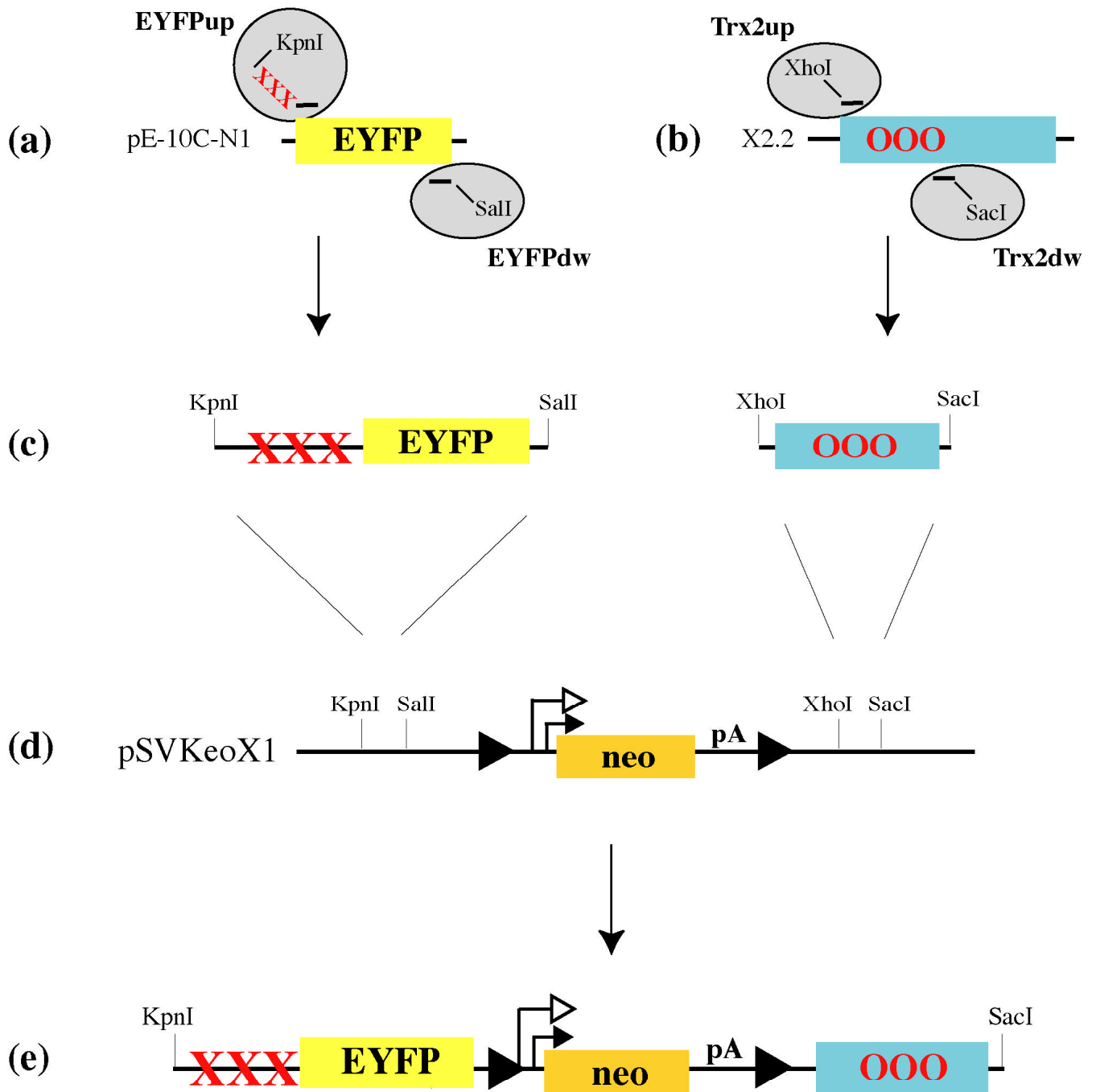
Figure 20: Cloning strategy for the targeting construct Fe13-EYFP-neo.
(a) PCR amplification of the EYFP coding region from expression plasmid pE-10C-N1 using oligos EYFPup and EYFPdw. (b) PCR amplification of the first 100 aminoacids of TRX2 (OOO) from the genomic subclone X2.2 using oligos Trx2up and Trx2dw. (c) Corresponding PCR products to the reaction in (a) on the left and the reaction in (b) on the right. (d) The plasmid pSVKeoX1 carries the neomycin resistance (neo) gene flanked by loxP sites (black triangles) as target sites for Cre mediated recombination. Neo is driven by a the eukaryotic SV40 early enhancer/promoter (white arrow) and the procaryotic beta-lactamase promoter (black arrow). Relevant restriction sites are indicated. (e) After conventional cloning of the two PCR products described in (c) into pSVKeoX1, arises the targeting cassette EYFP-neo. OOO and XXX are two homology arms that mediate ET/Red recombination into the Trx2 genomic subclone Fe13. This recombination event is described in detail in figure 21.

Cloning of this targeting cassette was performed in three steps (figure 20). The **first step** included a PCR amplification of the EYFP coding region from the EYFP expression plasmid pE-10C-N1 (*Clontech*) using the oligos EYFPup and EYFPdw (figure 20a). EYFPup contains 5' a KpnI restriction site, followed by a 60 bp sequence homologous to the Trx2 promoter region (XXX) including the initiating ATG, and the EYFP upstream PCR primer sequence not including the initiating ATG. EYFPdw contains 5' a SalI restriction site followed by the EYFP downstream PCR primer sequence not including the STOP codon. The **second step** included a PCR amplification of the first 100 amino acids of the Trx2 exon 1 not including the initiating ATG from the genomic sub clone X2.2 that spans the first exon sequence of Trx2 using the oligos Trx2up and Trx2dw (figure 20b). Trx2up contains 5' a XhoI restriction site followed by the Trx2 upstream PCR primer sequence not including the initiating ATG. Trx2dw contains 5' a SacI restriction site followed by the Trx2 downstream PCR primer sequence. In the **third step** corresponding PCR products (figure 20c) were cloned into the vector pSVKeoX1(figure 20d) that contains the neomycin resistance gene and polyadenylation signal flanked by lox P sites as target sites for Cre mediated recombination (Angrand et al., 1999) to create the targeting cassette EYFP-neo (figure 20e).

The linear KpnI/SacI fragment from EYFP-neo (figure 21a) and the Trx2 genomic sub clone Fe13 (figure 21b and 19c) were coelectroporated into electro-competent and arabinose-induced *E. coli* DH10B/pSC101/BAD/YZA cells to create the targeting construct Fe13-EYFP-neo by ET/Red recombination via the homology arms XXX and OOO (figure 21c and 19d). For detailed description of the ET/Red recombination procedure see results section R-1.1 and method section M-9.

In order to achieve a fusion of EYFP and the endogenous TRX2 protein, the targeting construct had to be stably integrated into the genomic locus. Electroporation of the linearized Fe13-EYFP-neo targeting vector was carried out into wt E14 ES cells (mouse line 129). To select for the homologous integration event cells were cultured in medium containing 250µg/ml G418.

To confirm the correct integration of the targeting construct into the endogenous locus, a southern strategy was developed (figure 22). Digesting with SalI enzyme and probing of the southern blot with an EYFP coding region fragment visualized the targeted band at 17
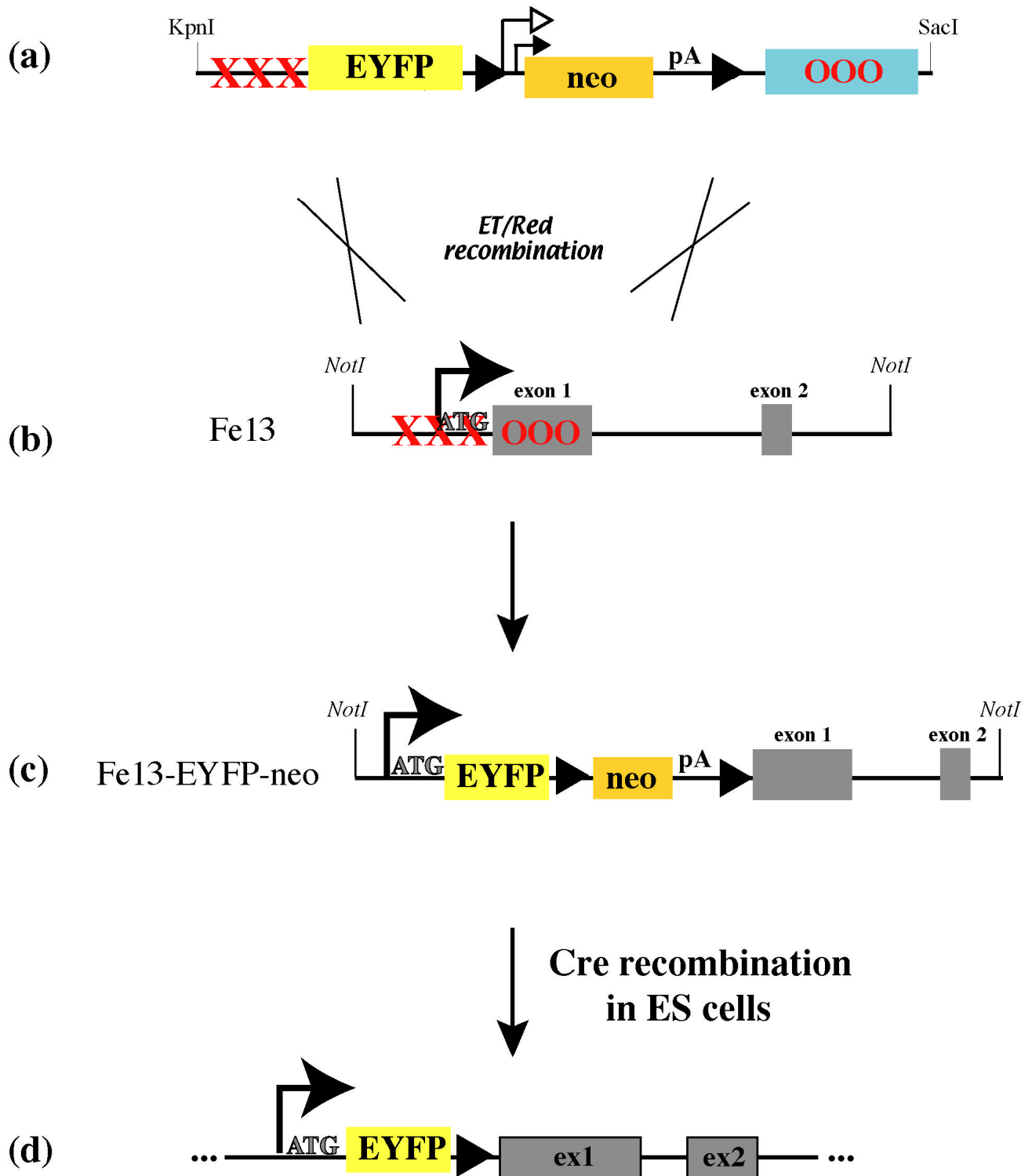
Figure 21: Creation of EYFP-TRX2 fusion in ES cells.
(a) The EYFP-neo targeting cassette contains the EYFP coding region, a neomycin selection marker (neo) flanked by loxP sites (=target sites for Cre recombination) followed by a polyadenylation signal (PolyA). Presence of a prokaryotic beta-lactamase promoter (white arrow) and eukaryotic SV40 early promoter/enhancer (small black arrow) ensures expression of neo in bacteria and ES cells. OOO and XXX are two homology arms that mediate ET/Red recombination into the Trx2 genomic subclone Fe13 displayed in (b) **Fe13** contains the endogenous Trx2 promoter (indicated by black arrow) and the first two exons of Trx2. (c) Fe13-EYFP-neo targeting construct after ET/Red recombination. (d) After homologous integration of this targeting construct into ES cells, Cre recombination leads to removal of the

kb and therefore confirmed the 5' integration side (figure 22d). To identify the correct 3' integration, genomic DNA digested EcoRI/SalI was probed with neo to confirm presence of the expected 12 kb fragment (figure 22e). Targeting the endogenous Trx2 locus with the EYFP-neo targeting construct could therefore be successfully confirmed. Southern analysis of DNA extracts from 96 stable transformants revealed a 15% rate of homologous integrations. Positive cell clones still contained the selection cassette including a polyA signal, and may therefore create a new heterozygous knock out allele of Trx2.

Only successful deletion of this selection marker by Cre mediated recombination would create the desired in-frame EYFP-TRX2 fusion. The stable EYFPneoTrx2 clone #22 was therefore transiently transformed with 40 µg of the Cre expression plasmid Mc-Cre and plated in low density (5000-50000 cells/ 10-cm-plate). Clones were picked in duplicates and plated with and without G418 selection. Clones that were non-resistant to neomycin were selected and screened for the recombination event by Southern analysis (figure 22f). In an EcoRI genomic digest probed with the BE580 fragment (annealing 3' of exon one) loss of the neo selection marker is documented by an about 1 kb size reduction of the targeted band.

8% of the analyzed clones showed the reduced   EcoRI fragment size specific for the neomycin deletion. Two clones (#95 and #70) were thawed from the 48-well-dish, expanded and frozen for further experiments.
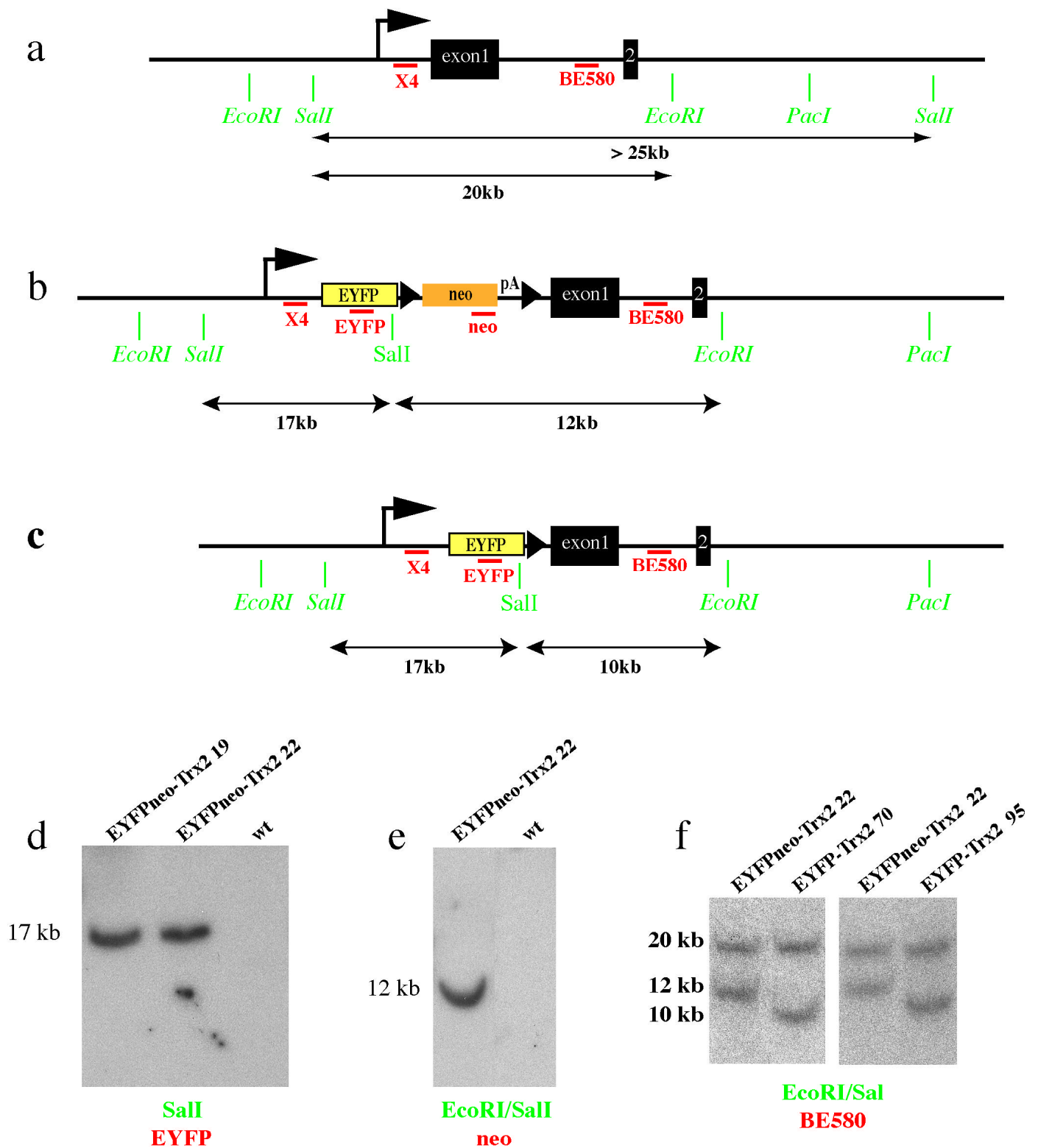
Figure 22: **Southern blot strategy and analysis of positive EYFP-Trx2 targeting in ES cells**.
Two Trx2 specific hybridization probes (X4, BE580), an EYFP specific and a neomycin specific hybridization probe are indicated in red. Genomic restriction sites (italics) and target construct specific sites (normal) are shown in green with the size of the digestion fragment indicated by two sided arrows. The wt allele (**a**), the EYFPneo targeted allele (**b**) and the EYFPneo targeted allele after Cre recombimation (**c**) are shown. (**d**) Wt and targeted cells before Cre recombination (clone EYFPneo-Trx 19&22) were SalI digested and probed with the EYFP probe. Both depicted clones show the expected 17 kb EYFP signal. (**e**) Homologous integration of EYFP-neo in clone EYFPneo-Trx2 22 is confirmed by the 12 kb fragment expected for the neo probe after EcoRI/SalI digestion. (**f**) Successful removal of neo upon Cre recombination in clone EYFP-Trx2 70&95) is documented by a 2 kb size reduction of the 12 kb targeted fragment upon Eco/Sal digestion and probing with BE580.

R-2.2 Sub cellular localization of EYFPtrx2

R-2.2.1   EYFP-TRX2 is a nuclear protein

Undifferentiated ES cells from the N-terminally labeled EYFP-Trx2 clones #95 and #70 were preincubated with a DNA stain for living cells (Hoechst) and analyzed using confocal microscopy to explore the distribution of endogenous TRX2 via the YFP fluorescent signal.

Undifferentiated stable EYFP-Trx2 ES cells show a weak but clearly nuclear yellow fluorescent signal, which suggests that endogenous TRX2 is a nuclear protein (figure 23a and b). Nucleoli as regions in which transcription of ribosomal genes is performed, generally do not show that same protein composition as the surrounding nuclear compartment (for review see Scheer and Hook, 1999) Since the EYFP-Trx2 fluorescent signal was excluded from nucleoli structures the localization could be regarded as specific. To further support this assumption and discriminate between auto-fluorescence and EYFP signal bleaching experiments were performed (data not shown). Since auto-fluorescence is not bleachable while EYFP fluorescence disappears after a long laser pulse (Chalfie et al., 1994) the high level of auto fluorescence could be discriminated from the Trx2 specific EYFP signal.

As additional information about TRX2 sub cellular localization behavior, the confocal analysis depicted that the EYFP-Trx2 signal was not detectable on mitotic DNA. As seen in figure 23, EYFP fluorescence is excluded from the nuclei of cells that are currently involved in mitotic divisions.

R-2.2.2   EYFP-TRX2 fades upon differentiation with RA

Since the initial perspective focused on the involvement of TRX2 in differentiation, efforts were taken to in vitro differentiate the targeted ES cells and investigate the dynamics of EYFP-TRX2 during the differentiation process. A very undirected and easy approach for ES in vitro differentiation was chosen namely deprivation of LIF, addition of retinoic acid (RA) 10^{-9} M to the differentiation media and cultivation of the ES cells without feeders. Unfortunately at this point it became obvious that survival of the 129 E14 ES cell line chosen for targeting is strictly dependent on feeder cells and that this ES
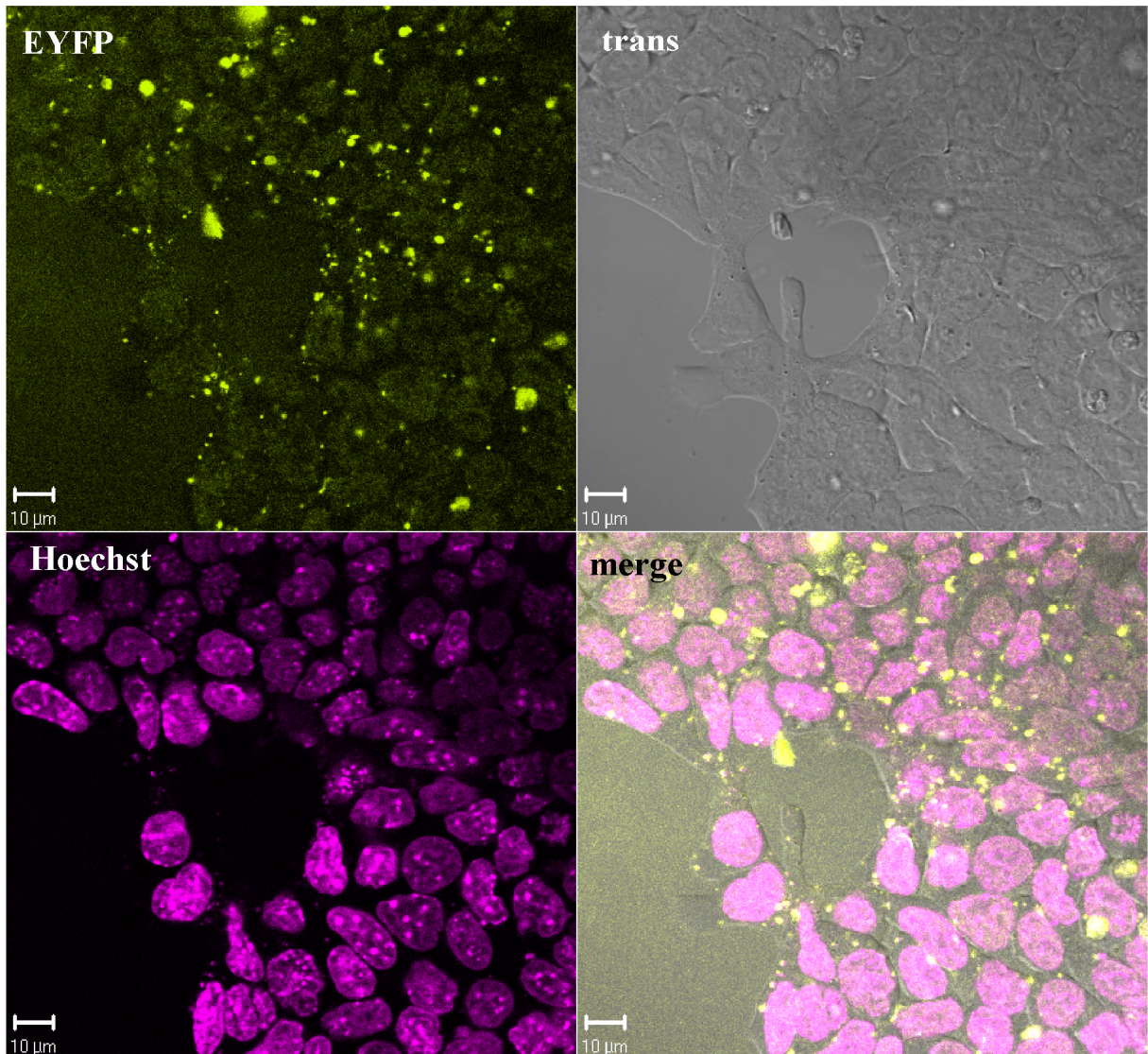
(a)

Figure 23a: Nuclear localization of EYFP-TRX2 fusion protein in ES cells.
Undifferentiated heterozygous EYFP-Trx2 clone 70 ES cells were alanyzed by confocal
fluorescence micsroscopy. Nuclear DNA staining by the Hoechste dye is shown in the bottom left
picture, while EYFP fluorescence is shown in the top left picture. The merge of both signals and
the transilluminatic view of the cell is shown in the bottom right and top right picture respectively
EYFP-TRX2 displays weak nuclear staining as indicated in the merge. Bright yellow speckles
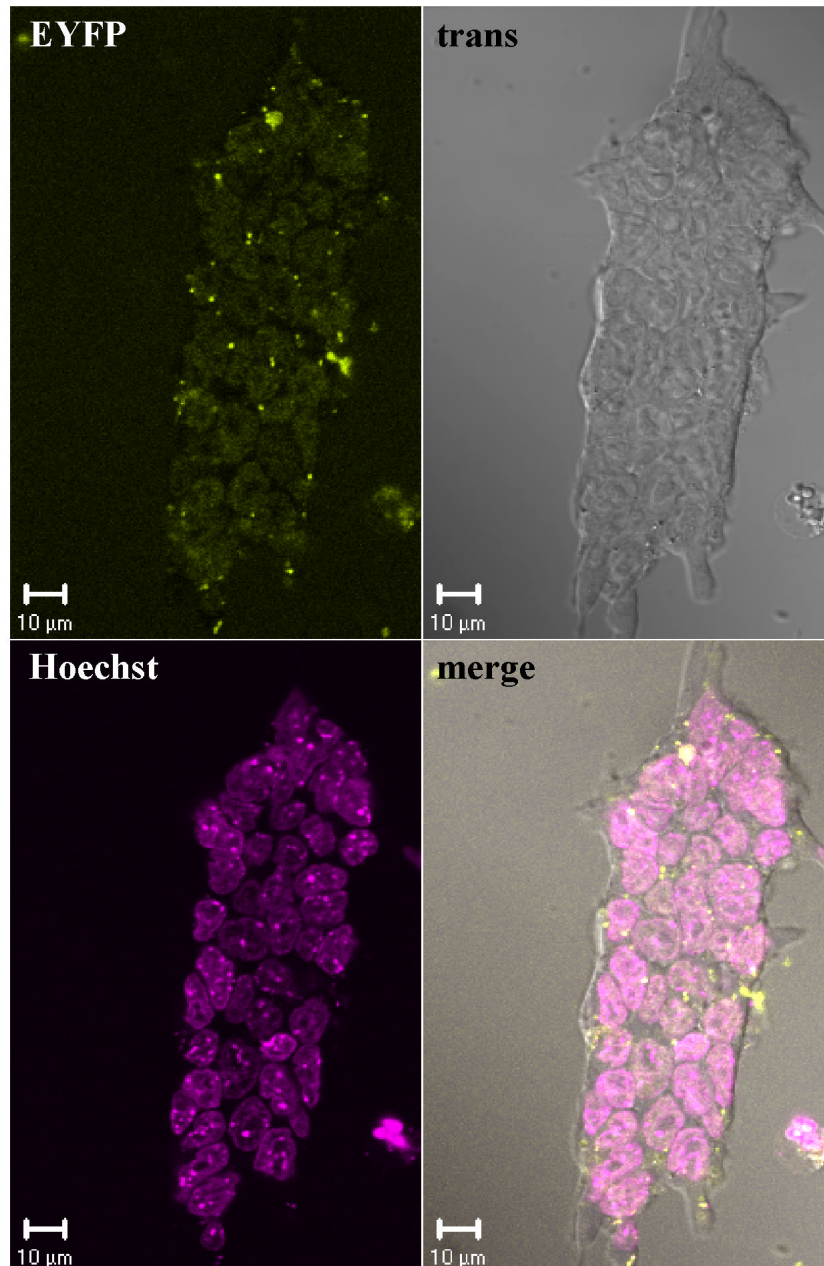were identified as unspecific staining by bleaching (not shown).

(b)

Figure 23b: Nuclear localization of EYFP-TRX2 fusion protein in ES cells.
Undifferentiated heterozygous EYFP-Trx2 clone 95 ES cells were alanyzed by confocal fluorescence micsroscopy. Nuclear DNA staining by the Hoechste dye is shown in the bottom left picture, while EYFP fluorescence is shown in the top left picture. The merge of both signals and the transilluminatic view of the cell is shown in the bottom right and top right picture respectively EYFP-TRX2 displays weak nuclear staining as indicated in the merge. Bright yellow speckles were identified as unspecific staining by bleaching (not shown).
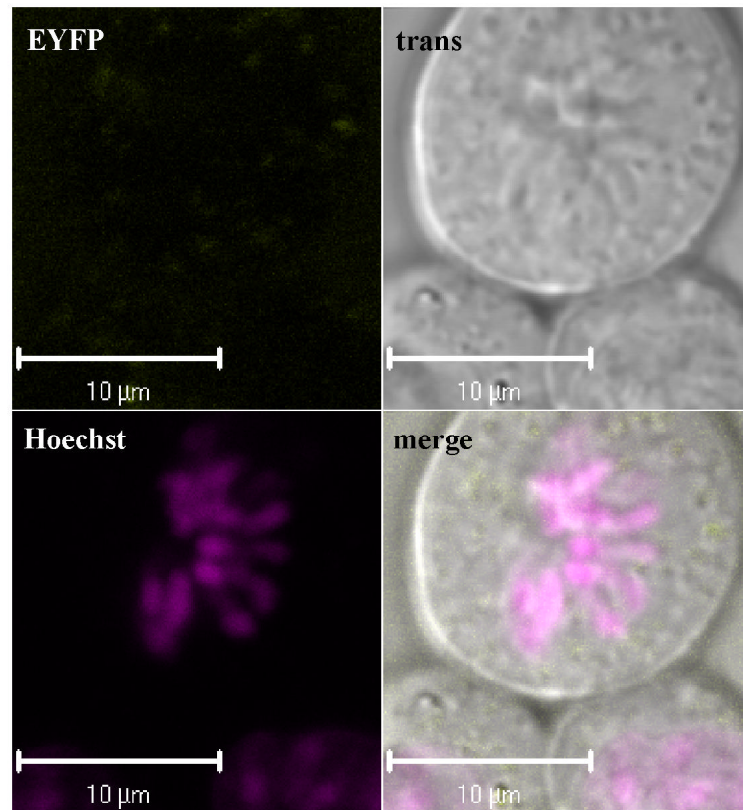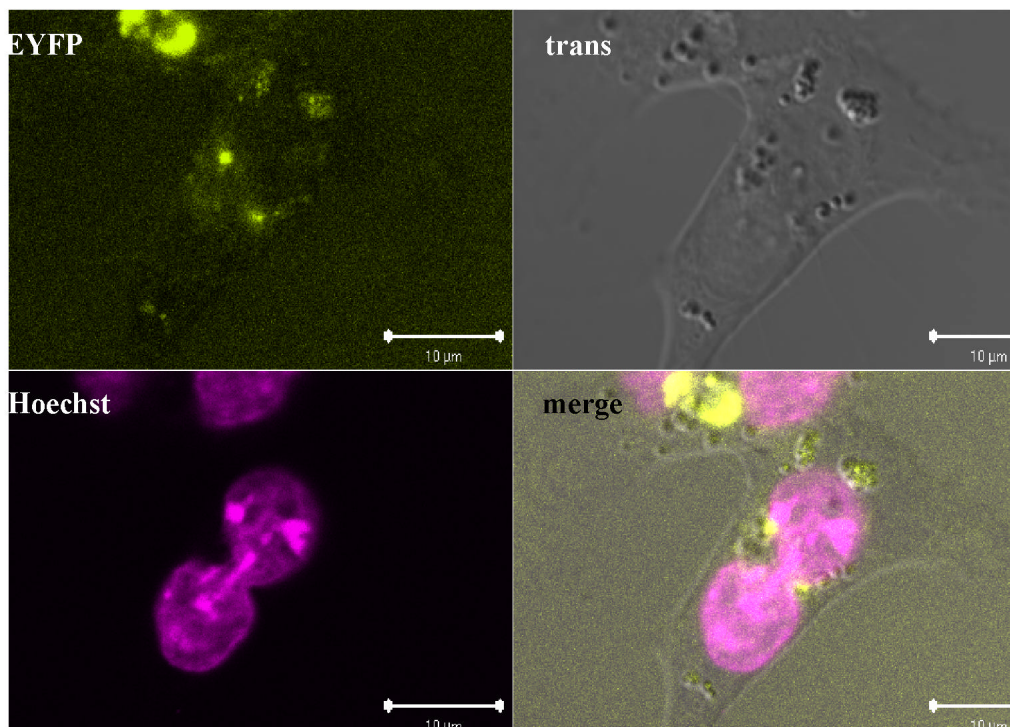
Figure 24: EYFP-Trx2 does not colocalize with mitotic DNA.
Undifferentiated heterozygous EYFP-Trx2 ES cells were alanyzed by confocal fluorescence micsroscopy. Nuclear DNA staining by the Hoechste dye is shown in the bottom left picture, while EYFP fluorescence is shown in the top left picture. The merge of both signals and the trasnilluminatic view of the cell is shown in the bottom right and top right picture respectively Cells depicted in (a) originate from clone EYFP-Trx2 #70 and in (b) from clone EYFP-Trx2 #95.

line is very hard if not impossible to differentiate. 80% of the cells detached from the culture dish within the first 12 hours of RA treatment, but analysis of the remaining 20% was continued. Figure 25 shows that the specific EYFP-TRX2 fluorescence in surviving healthy looking ES cells faded completely during a 36-hour differentiation process. To test whether this reflects the behavior of the endogenous TRX2 protein, a western on ES cells that carry a TRX2 N-terminal proteinA tag was performed. Tagged TRX2 heterozygous ES cells were treated with the same differentiation condition as previously the EYFP-Trx2 ES cells and crude protein extracts were loaded on a 15% Polyacrylamid gel to be blotted and probed with an anti-ProteinA antibody. Figure 25c shows that no decrease in TRX2 protein amounts caused by the differentiation procedure was observable. It was thus concluded that the disappearance of the fluorescent EYFP-TRX2 signal from differentiated ES cells did not reflect the natural behavior of the TRX2 protein. Removal of EYFP-TRX2 instead is an artificial effect created by the N-terminal fusion of EYFP to TRX2, which probably results in a modified TRX2 protein version that is unfavorable to the differentiating ES cell.

These experiments demonstrated that endogenous TRX2 is an exclusively nuclear protein absent from nucleoli structures, without any particular sub nuclear localization pattern. TRX2 protein is excluded from mitotic DNA.

R-2.3 Creation of EYFP-Trx2 mice

Since TRX2 was only localized dispersed in undifferentiated ES cells we argued that a specific localization patterns might only occur in differentiated tissue cells. For the investigation of this proposition in vitro differentiation protocols of EYFP-Trx2 cells were tried to achieve. Unfortunately the feeder dependent properties of the ES cell line used for targeting proved it impossible to differentiate ES cells in a reproducible manner. To be able to still access information about TRX2 distribution in differentiated tissue, the creation of a mouse line carrying the EYFP-TRX2 fusion protein was decided. Additional to the easy access to a variety of fully differentiated tissue, the mouse system could also
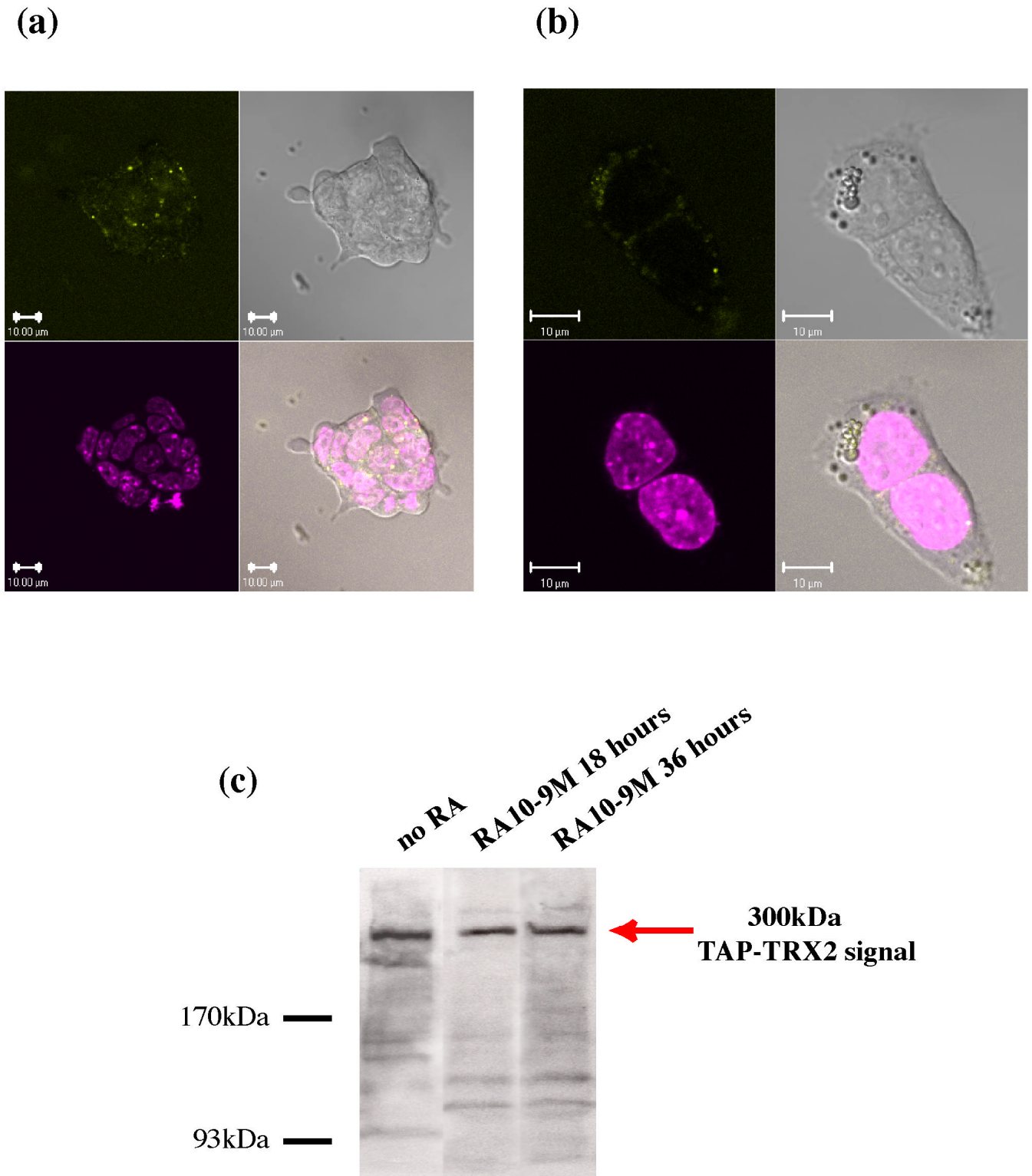
(a)

(b)

(c)

300kDa
TAP-TRX2 signal

170kDa

93kDa

Figure 25: **The EYFP-TRX2 signal fades upon differentiation with retinoic acid.**
(a) Confocal fluorescence analysis of EYFP-Trx2 heterozygous ES cells clone 95 after 1 day of treatment with 10-9M retinoic acid (RA) under differentiating conditions. **(b)** The same cell clone after 2 days of treatment with 10-9M retinoic acid. **(c)** Western analysis performed on crude protein extracts of heterozygous TAP-TRX2 expressing ES cells probed with ani-Protein A. ES cells were incubated with either no RA (left lane) in RA 10-9M for 18 hours (middle lane) or 36 hours (right lane). Since no RA induced diminishing of TAP-TRX2 can be observed, removal of EYFP-TRX2 from nuclear structures upon differentiation does not reflect the endogenous behaviour of TRX2 .

allow overcoming the problem of the weak fluorescent signal in heterozygous cells by creation of a homozygous EYFP-Trx2 line.

Cells from heterozygous clones EYFP-Trx2#95 and EYFP-Trx2#70 were injected into blastocysts derived from the mouse line C57BL/6. This specific mouse line allowed evaluating the germline transmission of the injected cells by hair color. The injected ES cell clones are derived from mouse line 129, which encodes for the dominant agouti hair color. If these ES cells contribute to the germline of the chimeric animals backcrosses of highly chimeric males to C57BL/6 wild type females should produce offspring with agouti hair color. To obtain germline transmission in blastocyst injection experiments the injected ES cells must be of exceptionally good quality. All backcrosses produced agouti hair colored litters indicating that both transgenic clones used gave germline transmission.

Since the possibility to identify the genotype of breeding progeny by hair color become too unreliable in further crosses, genotyping of coming litters was based by a PCR strategy performed on DNA tail preparations. As explained in figure 26a the use of primer L (annealing in the promoter region) and primer M (annealing in exon one) produces a fragment of 500 bp for the non-targeted wt Trx2 allele, and a fragment of 1000 bp for the targeted EYFP-Trx2 allele. While presence of both fragments therefore indicates a heterozygous animal, absence of the 500 bp fragment is characteristic for homozygous EYFP-Trx2 animals (figure 26b).

The heterozygous EYFP-TRX2 animals resulting from various crosses are without any apparent phenotype: live span, fertility and behavior is not altered compared to wt mice.

To obtain homozygous EYFP-Trx2 mice, heterozygous animals were crossed. Unfortunately the F1 generation of heterozygous crosses, failed to breed the expected Mendelian Ratio of 25% wt, 50% +/- and 25% homozygous pups. Instead only 6% EYFP-Trx2 homozygous pups survived until breeding age and into adulthood (table 3). Apparently the EYFP-TRX2 fusion created a hypomorphic allele for Trx2 with very heterogeneous penetrance that ranges from late fetal lethality to adult viability. Although the original aim was to use the fusion protein as a tool to study Trx2 localization in living cells, the hypomorphic phenotype presented the possibility to get further insight into Trx2 function and was therefore carefully analyzed.
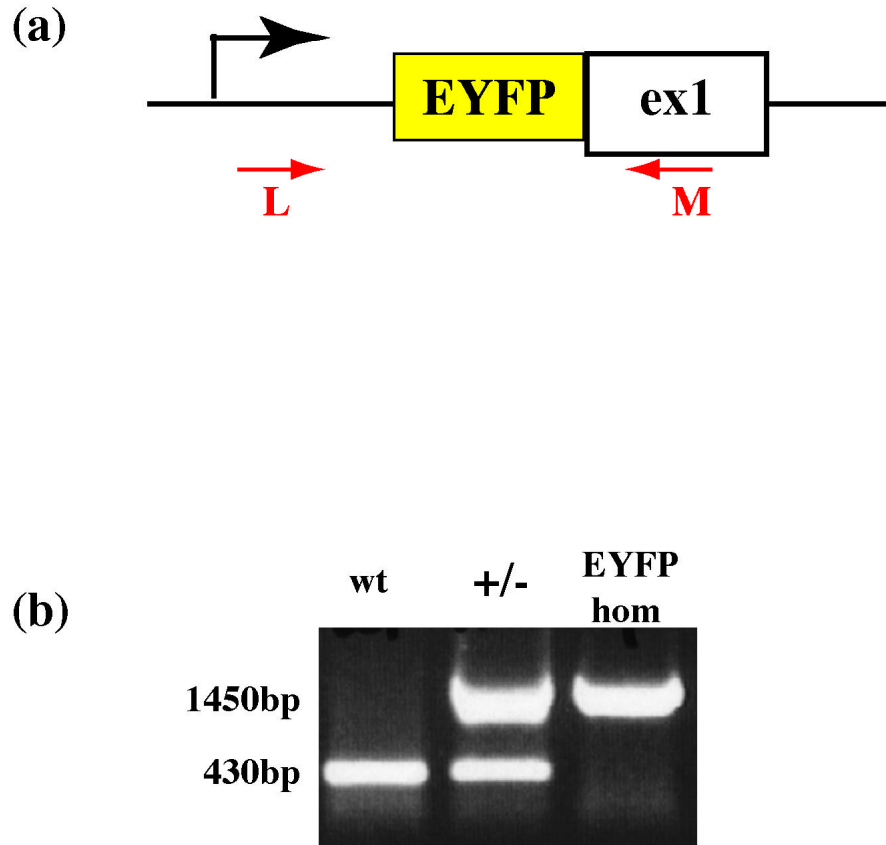
(a)

(b)

Figure 26: PCR based strategy to genotype mice for the EYFP-Trx2 mutation.
(a) Scematic deliniation of the position of primers L and M within the genomic locus of Trx2. Primer L anneales to the promoter region 5' of the EYFP coding region, primer M to sequence in the first Trx2 exon (ex1).   (b) The wt allele produces a PCR product of 430 bp. It is present in both wt (left lane) and heterozygous (middle lane) animals. The EYFP targeted allele produces a PCR product of 1450 bp since it includes the EYFP ORF of 1020 bp. Only this fragment is visible in genomic DNA from EYFP homozygous animals (right lane).

	EYFP #95		EYFP #70		Total	
wt pups	55	30%	36	38%	87	33%
+/- pups	104	62%	55	58%	159	61%
EYFP hom pups	13	8%	4	4%	17	6%
# of litters	32		18		50	

Table 3:   Litters from heterozygous EYFP-trx2 crosses.
Shown are the breeding results within 1 year between EYFP-trx2 +/- animals divided in +/- mice generated by blastocyste injection with cloneEYFPtrx2 #95, cloneEYFPtrx2 #70 and total. In Total 50 litters were genotyped to find a rate of only 6% for EYFP homozygous animals instead of the expected 25%.

stage analyzed	wt		+/-		EYFP hom		hom phenotype		Total
E8.5	8	23%	23	66%	4	11%	/	/	35
E10.5	17	33%	15	30%	19	37%	/	/	51
E12.5	9	27%	14	43%	10	30%	/	/	33
E14.5	13	31%	21	43%	12	26%	/	/	46
E17.5	12	26%	23	51%	11	24%	5	46%	46
E18.5	17	30%	31	54%	8	14%	7	85%	57
perinatal death	/	/	1	20%	4	80%	4	100%	5
weaning	51	30%	104	62%	13	8%	168		168

Table 4: Analyzed embryos to depict embryonic lethality of EYFP hom mice.
Litters from heterozygous crosses of EYFP-trx #95 were extracted and genotyped at various developmental stages. Numbers of wt, heterozygous and EYFP homozygous animals within those litters are listed in total numbers and percent. Until E14.5 no phenotypic abnormalities in EFFP homozyous animals is found. From E17.5 on EYFP homozygous embryos are significantly smaller than their litter mates and numbers drop from 25% to 14% (E18.5) and 8% (weaning age), suggesting that EYFP homozygous pups die perinatally because of reduced size and fittness through canibalism.

R-2.4 Analysis of the EYFP-Trx2 mutant phenotype

R-2.4.1 EYFP homozygous embryos are lethal upon birthing

To examine exact time point of embryonic lethality conveyed by the homozygous EYFP-TRX2 fusion litters from heterozygous crosses have been analyzed at E8.5, E10.5, E12.5, E14.5, E17.5 and E18.5. Table 4 summarizes the analysis of all EYFP-TRX2 heterozygous crosses. Only animals derived from blastocyst injections with the clone EYFP-Trx2 #95 in 50% C57BL/6 genetic background were used.

In litters examined at embryonic day E8.5, E10.5 and E12.5 no size difference between littermates of the three different genotypes (wt, heterozygous and homozygous) could be observed. The phenotype firstly appeared at around E17.5 when EYFP-Trx2 homozygous embryos are significantly smaller than their +/- and wt littermates (figure 26). At E17.5 46%, at E18.5 already 88% of the EYFP-Trx2 homozygous embryos show the observed growth retardation phenotype. The observed size variations could not be regarded as normal size heterogeneity within a litter since none of the wt or +/- littermates were ever growth retarded.

Until E17.5 the numbers of wt, +/- and EYFP-TRX2 homozygous embryos remain within the expected Mendelian Ratio of 25% / 50% / 25%, respectively. Up to birth EYFP-TRX2 homozygous numbers drop to 8%. Since these embryos still existed in the uterus of the mother shortly before birth (E18.5) but only 8% were found in the born litters it was concluded that reduced size and fitness of the EYFP-Trx2 pups induced cannibalism by the mother within the a few hours after birth. Four of the five pups which could be retrieved dead from the cage the morning after birth could be identified as EYFP-TRX2 homozygous embryos, which supported this assumption. Due to the speed and completeness of cannibalism by the mother it was not possible to genotype more pups that died perinatally.

It remains a topic of discussion whether the growth retardation in late embryonic development indicates a specific involvement of Trx2 in growth mechanisms or a non-specific consequence of generalized loss of gene expression.
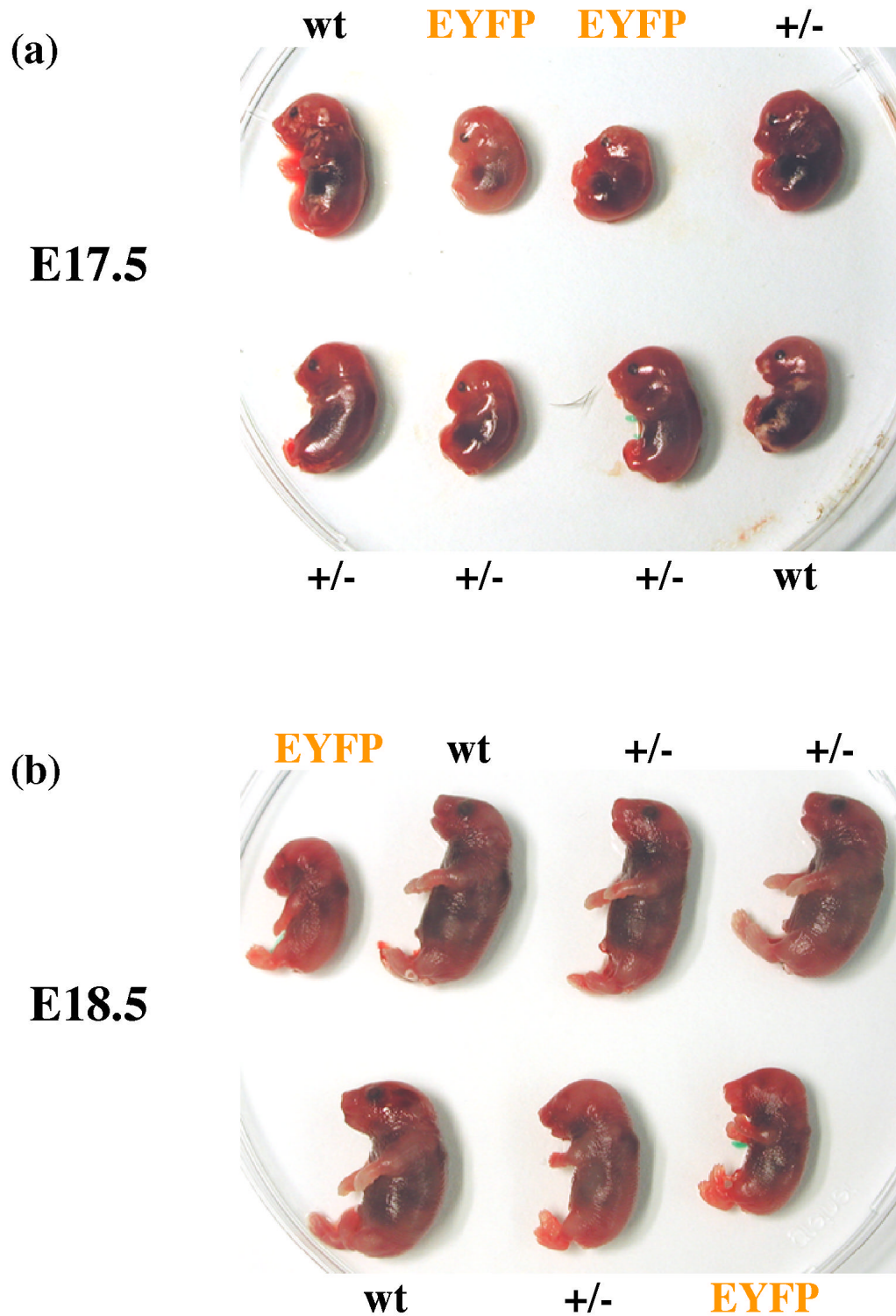
Figure 27: **EYFP-Trx2 homozygous embryos at E17.5 and E18.5 display a growth phenotype.** Homozygous EYFP-Trx2 embryos are significantly smaller than their heterozygous (+/-) or wt littermates at E17.5 (**a**) or E18.5 (**b**). Litters were obtained form heterozygous EYFP-Trx2 crosses.

R-2.4.2   Adult EYFP-Trx2 females are hypofertile

The adult EYFP-Trx2 homozygous survivors displayed no obvious phenotype compared to wt or +/- littermates. Until date no reduced life span (the oldest EYFP-Trx2 homozygous animals are now 22 months of age), size or weight differences could be observed.

To further investigate whether the exclusive expression of EYFP-TRX2 fusion protein in homozygous animals affects adult development, the surviving homozygous EYFP-TRX2 mice were tested for fertility by crossing them to wt animals of the same age.

All the tested males bred with a normal frequency (about one litter per month) and gave rise to normal size heterozygous litters of 5-8 pups. EYFP-Trx2 homozygous females revealed to be hypo fertile, as documented by prolonged breeding times and reduced litter sizes. In total only two of the eight homozygous females had one litter each with only three pups during a breeding period of one year. To examine this reduced fertility phenotype in further detail females were super ovulated by applying PMSG (pregnant mare serum gonadotropin) and HCG (human chorium gonadotropin) in an interval of 24 hours into the tail vein, and then bred with wild type males that had proven to be fertile. While all control heterozygous and wt females became pregnant, none of the homozygous females did. We concluded that the failure to conceive normally had to rest in the female reproductive organs and therefore examined the ovaries of super ovulated females. Examination was carried out by visually scoring for size and number of corpora lutea that represent ovulated eggs of the extracted ovaries.

Figure 27 shows that ovaries of EYFP-TRX2 homozygous females were not only generally smaller but also lacked the expected number of corpora lutea compared to ovaries from wt females, indicating a failure of ovulation.

All above results demonstrate that the EYFP-Trx2 mutation creates a hypomorphic allele. The phenotype of EYFP homozygous mice involves perinatal lethality and diversely severe hypo fertility in the surviving females.
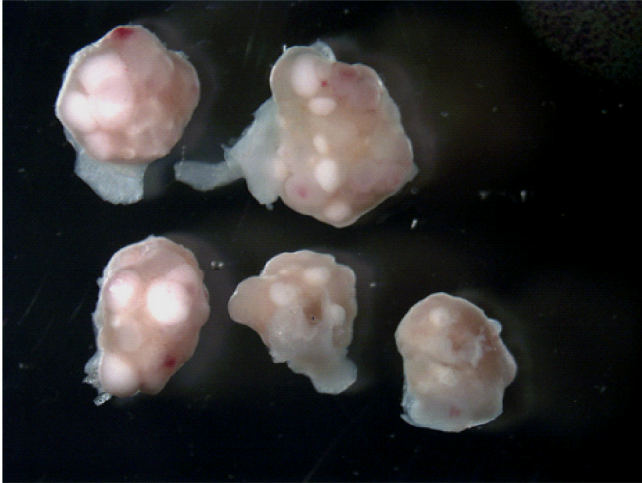
wt   **EYFP hom**
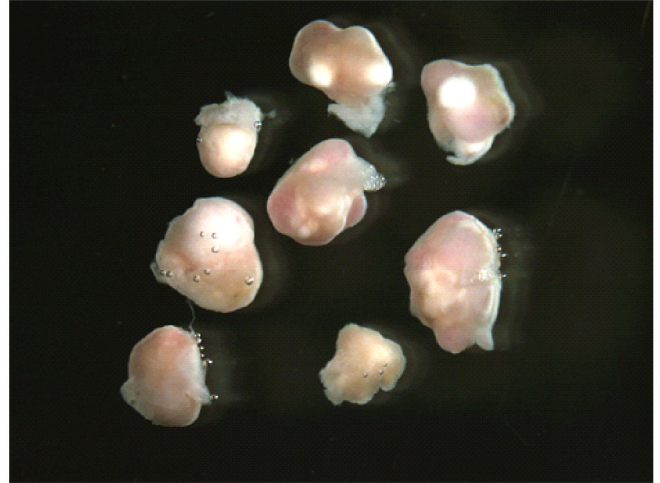
comparison

**Figure 28:   Ovaries from EYFP-Trx2 homozygous females are retarded.**
Ovaries from 40-week old females were extracted and compared for size differences and number of corpi albae. EYFP-Trx2 homozygous females have generally smaller ovaries and less Corpi albae a phenotype that could explain the observed hypofertility caused by the sole expression of the modified, EYFP-targeted version of TRX2 protein..

R-2.5 Trx2 mRNA levels in EYFP-Trx2 heterozygous ES cells are reduced

To look for a molecular explanation of the EYFP-Trx2 hypomorphic allele a northern blot analysis on RNA extracts from the two independent EYFP-Trx2 ES cell clones #95 and #70 that went germline was performed. This revealed that mRNA levels of the fusion transcript are observably reduced compared to wt Trx2 mRNA levels. In figure 29, 30 µg total RNA from wt, Trx2 +/-, Trx2 -/- and two independent heterozygous EYFP-Trx2 ES cell clones were separated on formaldehyde containing agarose gel and transferred to nitrocellulose membrane. Blots were probed with the Trx2 specific hybridization fragment CF14 and an EYFP specific hybridization fragment while an actin hybridization probe served as an internal loading control. Additionally double (60 µg) and half (15 µg) amounts of total RNA from EYFP-Trx2 ES cells were loaded for better comparison. The signal was quantified by phosphoimager evaluation (figure 29b). 60 µg of total RNA from EYFP-Trx2 ES cells and 30 µg of wt total RNA led to comparable CF14 signal intensities. 30 µg of total RNA from Trx2 heterozygous ES cells gave comparable CF14 signal intensities as 30 µg of total RNA from EYFP-Trx2 ES cells. 15 µg of total RNA from EYFP-Trx2 ES cells gave halved CF14 signal intensities compared to the signal in 30 µg of total RNA from Trx2 heterozygous ES cells. Since Trx2 mRNA levels in heterozygous EYFP-Trx2 expressing ES cells are therefore comparable to the Trx2 mRNA levels of ES cells carrying a heterozygous knock-out of Trx2, it was concluded that only very limited amounts of EYFP-Trx2 mRNA were produced. Probing of the same blot with an EYFP specific probe though visualized a fragment of similar size as the fragment visualized by CF14 hybridization, thus confirming that at least a sub-fraction of EYFP-Trx2 RNA is present in heterozygous EYFP-Trx2 ES cells.

These results suggest that the observed phenotype in EYFP-Trx2 homozygous mice is probably due to overall reduced TRX2 levels caused by a defect in transcription or RNA stability.

(a)
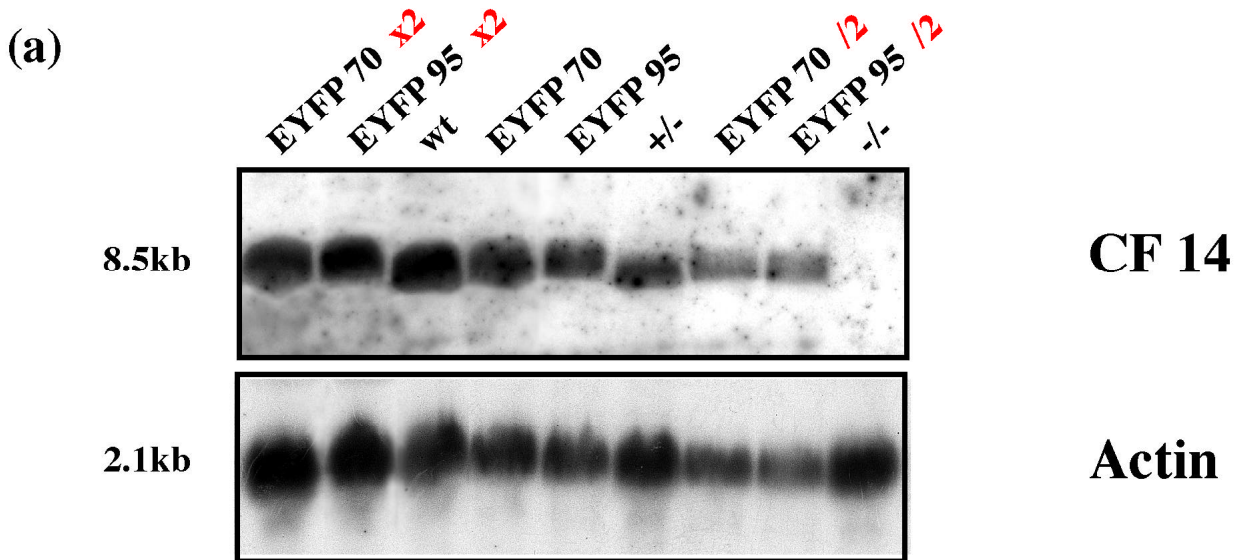
(b)

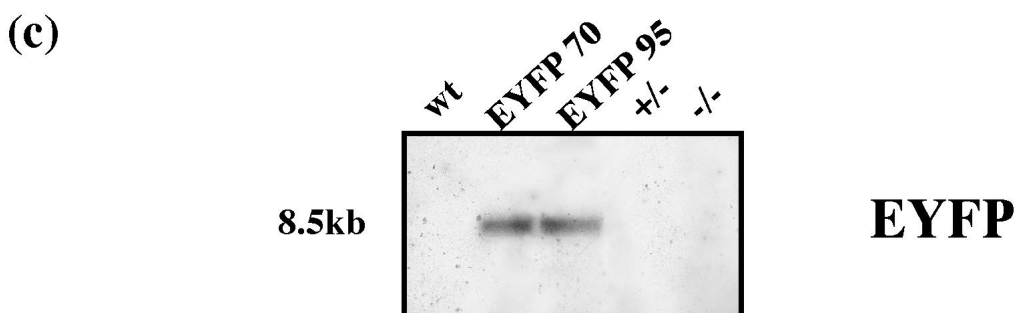|	Area [pixels]	Actin signal (-Bkg) [a.u.]	CF14 signal (-Bkg) [a.u.]
EYFP 70 x2	580	725.4	64.3
EYFP 95 x2	580	708.6	56.6
wt	580	406.6	62.3
EYFP 70	580	428.7	49.7
EYFP 95	580	389.9	43.3
+/-	580	426.6	40.2
EYFP 70 /2	580	207.8	23.4
EYFP 95 /2	580	172.8	23.1
-/-	580	447.6	-1.4

(c)

Figute 29:   Trx2 mRNA levels in EYFP-Trx2 heterozygous ES cells are reduced.
(a) Total ES cell RNA from two heterozygous EYFP-Trx2 clones (#70 & #95) was quantitatively compared to RNA from wt, Trx2 +/- or Trx2 -/-. 30 yg RNA was loaded for wt, Trx2 +/-, Trx2 -/- and for the EYFP-Trx2 clones #70 & #95. Additionally double amounts (x2 = 60yg) and half amounts (/2= 15yg) total RNA of EYFP-Trx2 clones #70 & clone #95 were loaded for better comparison. Actin was used as a loading control.   **(b)** Phosphoimager quantification of signals detected in (a) supports that CF14 detected Trx2 mRNA levels in heterozygous EYFP-Trx2 cells is reduced to about half.   **(c)** Probing with the specific EYFP probe reveals EYFP-Trx2 transcripts exclusively in EYFP targeted ES cells. EYFPtrx2 transcripts run at same position as Trx2 trasncripts, suggesting that targeted RNA is transcribed in full size and that at least a subfraction of EYFP-Trx2 mRNA is produced..

R-2.6 TRX2 protein is reduced in EYFP-Trx2 heterozygous ES cells

To confirm that reduced EYFP-TRX2 RNA levels are also mirrored on the protein level, western blot analysis using the TRX2 specific antibody was performed on ES cell extracts (figure 30).

Crude protein extracts from wt, Trx2 -/- and the two independent heterozygous EYFP-Trx2 ES cell lines were separated by 5% SDS-PAGE, transferred to nitrocellulose and probed with the specific TRX2 antibody. Probing with an actin specific probe served as an internal loading control.

Probing with anti-TRX2 detected a highly degraded but Trx2-specific pattern in the wt sample as well as in EYFP-Trx2 ES cell extracts. Even though the same amount of cells were extracted and same amounts were loaded on the gel as confirmed by the comparable intensities of the actin signals, due to the high level of degradation a quantitative comparison was very difficult. It seems as if TRX2 protein levels in EYFP-TRX2 #95 ES cells is reduced compared to wt cells while the signal reduction is much stronger in EYFP-TRX2 #70 ES cells. Since this is a subjective measure it can only be assumed that TRX2 protein levels are reduced in EYFP-TRX2 ES cells compared to wt ES cells.
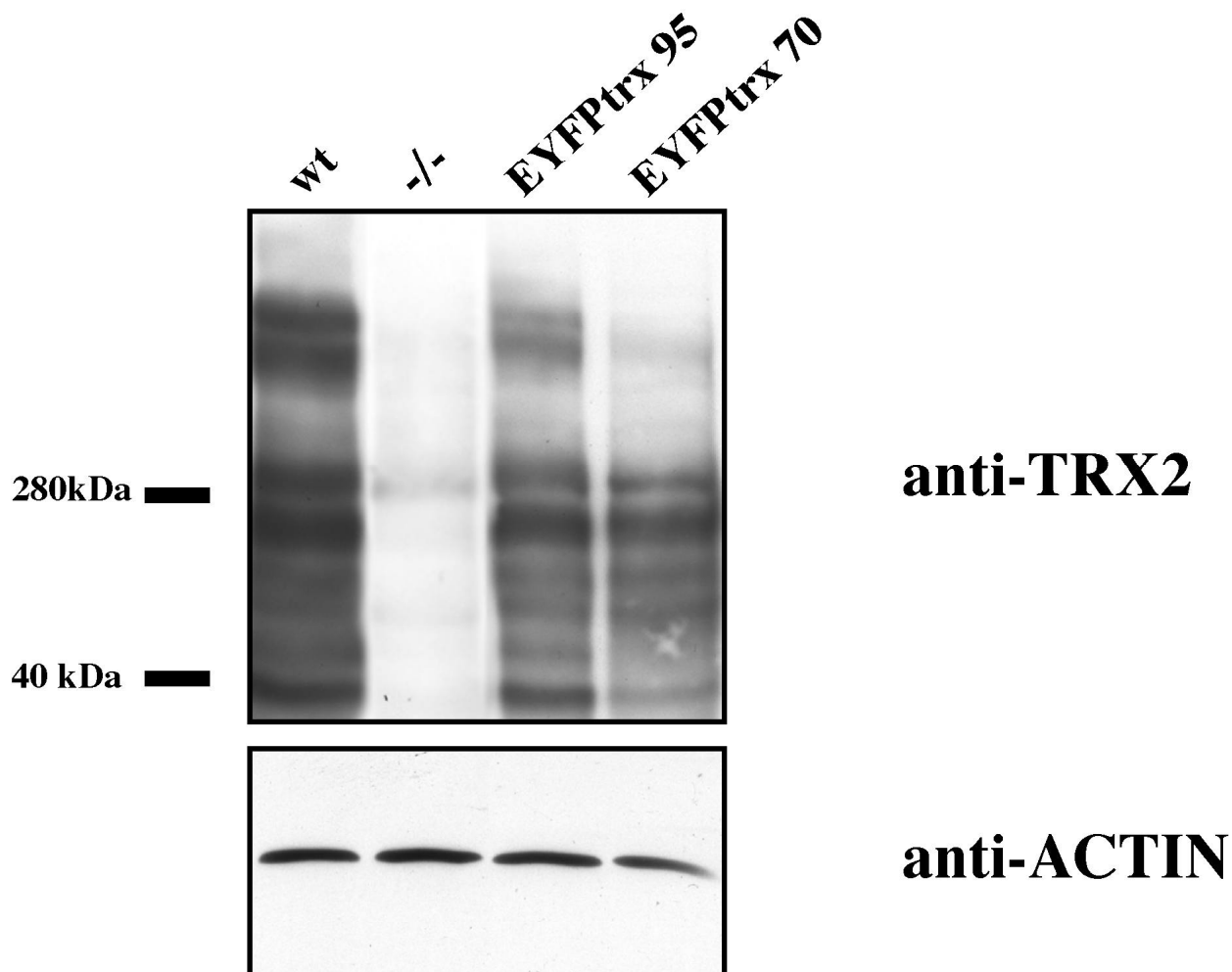
Figure 30:   TRX2 protein is reduced in EYFP-Trx2 +/- ES cells..
Specific anti-TRX2 antibody detects the 280 kD TRX2 signal and degradation products in crude
extracts of wt, and two independent EYFP targeted (clone 70 and clone 95) ES cells. The internal
loading control (ACTIN probe) shows that comparable amounts of total protein were loaded. Even
though a very high grade of degradation obscures the quantification, it seems as if the TRX2 signal in
EYFP targeted ES cells is reduced compared to wt. Molecular weights in kD are indicated left of the
membrane.

D. Discussion

D-1   TRTX2 is nuclear and absent from mitotic DNA

Fluorescence microscopy of the EYFP-Trx2 ES cells showed a nuclear localization of the chimeric protein. The nuclear distribution could be described as diffuse, no specific sub nuclear pattern was observed. This observation is in contradiction to the performance of TRX2's ortholog MLL, which has been reported to localize to discrete nuclear speckles, a manner accredited to the SNL sequences in the N-terminus of the protein (Yano et al., 1997; Caslini et al., 2000; Lee and Skalnik, 2002). Also another SNL containing protein CGBP (human **C**p**G B**inding **P**rotein) shows the nuclear punctuate distribution and is even co localized to MLL containing speckles (Lee and Skalnik, 2002). MLL and CGBP associate almost exclusively with transcriptionally active euchromatin, and with the nuclear matrix suggesting a role in modulation of chromatin structure, leading to epigenetic effects on the maintenance of gene expression. Even though SNL sequences are also present in TRX2 this punctuate distribution could not be reproduced for EYFP-TRX2 in ES cells. Whether this reflects the natural behaviour of TRX2 in the totipotent context of ES cells or is ascribed to the disturbing presence of the EYFP fusion remains ambiguous.

Unlike MLL, EYFP-TRX2 fluorescence was also not found on metaphase DNA, a comportment shared with CGBP (Caslini et al., 2000; Lee and Skalnik, 2002). This disparate sub-cellular localization during cell division suggests that TRX2 (and also CGBP localization) is a dynamic process that undergoes regulation through the cell cycle or upon differentiation.

D-2   TRX2 requirement in ES cells

Observations from the cell culture experiments performed on TRX2 deficient ES cells can be summarized with the conclusion that TRX2 is not required for ES cell viability. Targeting the second allele of Trx2 was successful. Targeting frequencies for the first allele (performed by v.d. Hoeven) were around 9 % (our unpublished results). The second allele was targeted with the similar frequency of 3%, taking into account that only one allele was available for the desired targeting and double knock out. Trx2 knock out ES cells grew with the same doubling time and cell cycle distribution as +/- and wt cells, are unobtrusive with respect to apoptosis rates and retain the ability to form wt colonies. Therefore Trx2 is not required for an essential or apparent cell cycle function in ES cells that represent embryonic stage E4.5. This conclusion is supported by the mouse knock out phenotype and blastocyst injection experiments with Trx2 -/- ES cells, since before E7.5 a phenotypic abnormality of k.o. embryos or high chimeras has not been observed. However studies in *Drosophila* have provided evidence for a maternal supply of TRX. The small 10 kb trx2 mRNA is exclusively found in adult females and 0-3 hour embryos and was therefore classified as of maternal origin (Mozer and Dawid, 1989; Breen and Harte, 1991; Sedkov et al., 1994). We therefore have to consider that maternally supplied Trx2 from the heterozygous oocyte provides some early functions of Trx2.

However the ES cell studies exclude TRX2 as a factor involved in general processes like the cell cycle or housekeeping-gene regulation. ES cells are totipotent cells derived from the inner cells mass of the blastocyst. Since also our targeted -/- ES cell line retained the potential to contribute to many embryonal tissues (proven by the blastocyst injection experiment), it can be regarded as undifferentiated. Apparently TRX2 is not important for viability of the cell in an undifferentiated state, but plays an important role in later developmental stages indicating that it might be differentiation processes that require TRX2 function. In vitro differentiation studies on Trx2-/- versus wt ES cells are currently being investigated.

D-3   EYFP-TRX2 hypomorphic phenotype

EYFP-Trx2 homozygous animals were not born at the expected Mendelian ratios. Also EYFP-Trx2 female homozygous survivors were infertile. This oberservations could indicate a toxic effect of the created fusion protein. This toxicity cannot be contributed to the sole presence of EYFP since no toxic or negative effect for EYFP both in differentiated mammalian living cells (Ellenberg et al., 1998) or in the mouse itself (Hadjantonakis et al., 2001, Hadjantonakis et al., 2002; Metzger et al., 2002) has been reported. The mouse produced from Hadjantonakis, for example, conducts a widespread and strong expression of EYFP and remains vital and fertile. Also the use of EYFP in fusion with a protein of interest has proven to leave the wt function of the protein unaffected. In this context neither C-terminal fusions of a fluorescent protein (Godwin et al., 1998, Kanda et al., 1998; Ellenberg et al., 1997; Presley et al., 1997, Drew et al., 2001) nor N-terminal fusions (Baumann et al., 1998; Stradal et al., 2001) make a difference. The expression of fluorescent proteins in mammalian systems therefore remains a powerful tool for the study of intracellular processes.

Instead, we conclude that addition of EYFP to Trx2 interferes with either the normal function of the endogenous TRX2 protein or with the molecular processes for the production of the protein. Analysis of EYFP-Trx2 transcription and protein levels suggest a defect with the latter since Trx2 mRNA in EYFP-Trx2 heterozygous ES cells is found in reduced amounts. However the EYFP-Trx2 mRNA is detectable in +/- ES cells and also confocal fluorescence microscopy could specifically detect the presence of the fluorescent fusion protein, indicating that at least a sub fraction of EYFP-TRX2 is indeed produced in ES cells. Hence we conclude that the phenotypic impact of the EYFP-Trx2 allele is at least partly due to reduced mRNA levels. This could be either due to decreased transcription or decreased RNA processing and/or stability. These matters were not investigated, however it is worthy noting that Trx2 transcription, like Mll, arises in a CpG island, which encompasses the initiating methionine. Hence the EYFP coding region was inserted into the CpG island, albeit near its C-terminus.

The observation that EYFP-TRX2 is undetectable after differentiation of ES cells (an observation that is not reflected by the endogenous protein behaviour) could either indicate that TRX2 in fusion with EYFP retains some abnormal protein features that is

toxic for the differentiating cell or that upon differentiation the expression changes in a way that the already impaired EYFP-Trx2 expression is further impaired. In any case, these features of EYFP-TRX2 precluded insights into the natural sub cellular localization of TRX2, however did provide a hypomorphic allele for analysis during development.

If the EYFP-Trx2 allele interfered with both mRNA expression and protein function, the negative influence of the EYFP fusion on the protein level could be an improper folding mechanism or the interference of the EYFP attachment with N-terminal domains of TRX2 namely AT hooks, SNL sequences and MTase domain. As mentioned in the introduction the combined actions of those domains could contribute to the ability of TRX2 to recognize, bind and therefore maintain previously activated promoter regions in an active state. If the EYFP fusion has a negative effect on the function of those domains, loss of this important TRX2 feature could lead to the replacement of the impaired protein version with the wt copy present in heterozygous ES cells and mice. Since also a heterozygous k.o. of TRX2 in mice and ES cells has no phenotypic effect (our unpublished results), presence of the EYFP-Trx2 allele can remain phenotypically unobtrusive until also the second (wt) copy of Trx2 is lost upon production of homozygous animals.

The fact that adult survivors that carry a homozygous EYFP-Trx2 allele exist can only be explained by the presence of a second site mutation or some other kind of adaptation in those animals that balanced the deleterious alleles to an extent that allows normal development at least with respect to fundamental vital functions and life span. Still even in adult EYFP-Trx2 homozygous survivors TRX2 impairment resulted in an irregular development of the reproductive female organs, so that we decided that unfortunately also EYFP-Trx2 homozygous mice could not be drawn to analyse the wt features of TRX2 protein.

We conclude that the phenotype of EYFP-Trx2 homozygous mice is probably caused by reduced TRX2 levels due to impaired expression, but cannot exclude a contribution from impaired TRX2 function due to the N-terminal fusion of EYFP.

D-4   Continuous need of TRX2 during development

Our original intention to use the EYFP-TRX2 fusion as a tool to study dynamic changes of TRX2 sub cellular distribution before and during differentiation failed. Hence our efforts focused on the analysis of the hypomorphic phenotype. While other work so far has centred on investigating the early functions of TRX during embryonic development, this hypomorphic Trx2 allele allows insights also into some of the late functions of the gene. In early development no phenotype was evident while EYFP-Trx2 homozygous embryos of developmental stage E17.5 and E18.5 were significantly smaller than their heterozygous and wt littermates. Until E18.5 the numbers of wt, +/- and EYFP hom embryos remained in the expected Mendelian ratio. After birth EYFP homozygous numbers drop to 4%. We conclude that reduced size and fitness of the EYFP-Trx2 pups caused cannibalism by the mother within the a few hours after birth. Whether this indicates a specific involvement of Trx2 in growth mechanisms or a non-specific consequence of generalised loss of gene expression remains a topic of discussion but allows the conclusion that TRX2 is indeed involved in gene expression control during fetal stages. Breeding of the 4% EYFP-Trx2 homozygous surviving animals revealed that the males are fertile, whereas the females are hypofertile, an observation that led to the analysis of the female reproductive organs. Studies on ovaries of EYFP-Trx2 homozygous females revealed both a lack of corpora lutea as well as a generally reduced size. The aberrations indicate that TRX2 is also involved in the very late developmental process of female reproductive organ formation.

The EYFP hypomorphic allele therefore indicates that Trx2 function is not only required in an early state of development (that is between E7.5 and E9.5 when the -/- embryos die), but that also at late stages of development suggesting that TRX2 function is broadly and continuously required.

D-5   TRX2 is required in all cell types

In lower and moderate chimeras for Trx2-/-, Tx2 deficient cells are still observable in all tissues around E10.5. All the chimeras that show a more or less dispersed finding of -/- cells appear developmentally inconspicuous indicating that there is a threshold, under which -/- cell contribution can be tolerated without disturbing normal early embryonic development. As soon as the number of -/- cells is above this threshold (high content chimeras and homozygous knock out embryos), animals are retarded as discussed above. However analysis of later low chimeric, phenotype-less stages show that Trx2-/- cells are progressively eliminated until birth (E18.5). This points to the presence of developmental processes in the late embryo that do not tolerate any participation of Trx2 deficient cells.

Supported by analysis of the phenotype of EYFP-Trx2 homozygous survivors (D-4), and low chimeric Trx2-/- injection chimeras (D-5) we can conclude a broad and continuous need for Trx2 even in later stages of development without any indication of cell type specificity.

D-6   Is TRX2 required for differentiation processes ?

The last areas in which Trx2-/- lacZ-expressing cells could still be observed by E18.5 include macrophages and/or osteoblasts, in cartilaginous areas forming bone. Bone development is described as occurring by two different processes: intramembranous (in membrane) ossification of the facial and cranial bones and endochondral (in cartilage) ossification characteristic for long bone formation of the axial and limb skeleton. Intramembranous ossification involves direct osteogenesis from undifferentiated mesenchyme without any cartilage precursors while endochondral ossification requires a cartilaginous model resembling the future bone in shape and relative size. In both processes osteoblasts are the bone-forming cells that form ossification centres by deposition of collagenous fibres and calcium salts as the organic matrix of the bone (osteoid). Osteoblasts differentiate from undifferentiated mesenchymal cells; whether in

the case of endochondral ossification, osteoblasts differentiate from cartilage cells or simply replace previously removed chondrocytes remains unclear. Although both ossification methods can be separated in analysis, long bones serve as examples of both processes: a collar of bone surrounding the cartilage model is laid down by intramembranous ossification while inner-bone ossification by endochondral pathways take place. Therefore in most bone developmental processes, intramembranous ossification is involved (Hamilton et al., 1972; Mundlos and Olsen, 1997; Cohen, 2000). This differentiation process of bone formation is one of the last in mouse development. First signs of ossification appear around E14.5 in the mandibular and maxillar regions of the facial skeleton. Only by E19.5 the ossification process is completely termed. Ossification in long bones of the phalanges is a late event in development starting at E18.5 and still being continued postnatally (Kaufmann, 1992).

Macrophages are involved in the cellular immune reaction but also play a role in the bone formation process. Macrophages and their specialized cell-type the osteoclasts, are responsible for the removal of cartilage cells before their replacement with osteoblasts.

Since TRX2 does not appear to be required in ES cells and TRX2 -/- cells are eliminated during development, it may be that TRX2 is required for differentiation processes. If so, then the perdurance of Trx2-/- cells at sites of ossification may reflect the possibility that cells at these sites are amongst the last areas to require commitment to a differentiated condition. The fact that TRX2 absence is only tolerated in so far undifferentiated cells, again indicates that Trx2 plays an important role in tissue differentiation.

Together with the observations of the EYFP hypomorphic phenotype, the results show that Trx2 is continuously required throughout development. Furthermore the chimeric analysis failed to show any notable cell-type specificity, so we can conclude that Trx2 function is required in all cell-types, or the lack of Trx2 provokes apoptosis in all cell-types.

D-7   Support for the Trx2 k.o. mouse phenotype

As described in the introduction the phenotypic analysis of Trx2 deficient embryos revealed a retardation in size and developmental stage from embryonic day E7.5 on. The very general nature and the timing of that retardation would also include the possibility that it was caused by a defect in the extra embryonic tissue. The extra embryonic tissue more precisely the polar trophectoderm has important assignments in the process of implantation of the embryo into the uterus wall from embryonic day E4.5 on. Especially embryonic stage E7.5 (T11) is characterized by the development of extra embryonic structures like yolk sac, chorion or allantois whose prime role is to provide physiological and nutritional environment for the developing embryo, but who are also involved in the supply of red blood cells and primordial germ cells an accomplishment that is crucial for embryogenesis (Kaufmann and Bard, 1999). Furthermore the extra embryonic visceral endoderm plays a key role in the developmental patterning of the underlying ectodermal tissue (epiblast) (Beddington and Robertson, 1998). Gene knockouts that generate an extra embryonic tissue defect, display phenotypes similar to the one of Trx2 with respect to timing and embryonic retardations (Zeitlin et al., 1995, Dragatsis et al., 1998; Yamamoto et al., 1998; Meagler and Brown, 2001).

The -/- ES blastocyst injection experiments exclude the possibility that the Trx2-/- phenotype is due defects in the extra embryonic tissue. Cells from the inner cell mass of a blastocyst and therefore also the injected Trx2-/- ES cells can only contribute to the embryo proper not to the extra embryonic tissue (Kaufmann and Bard, 1999). Extra embryonic tissue in those embryos is completely formed by wt cells of the host C57BL/6 blastocyst so that the retarded phenotype of chimeras with a high contribution of -/- cells that is very similar to the phenotype of Trx2 deficient embryos, is entirely caused by the lack of TRX2 in the embryo itself.

The fact that the high content chimeras mirror the phenotype of knock out embryos therefore implies that Trx2 function is intrinsic and not due to the lack of Trx2 in extra embryonic tissue. There is a cell autonomous need for TRX2 in the epiblast cell.

D-8   Outlook

D-8.1   Is Trx2 involved in in-vitro differentiation ?

In chapter R-1.5 I was able to show that the Trx2 deficient ES cell line did not show a defect in either proliferation, cell cycle distribution, colony formation or apoptosis rates and concluded that Trx2 is not an essential gene for the cell in the pluripotent undifferentiated ES cell state. This observation is raising the question about a possible Trx2 involvement in differentiation. To investigate this topic, in vitro differentiation of ES cells can be applied. Possibly the knockout of both Trx2 alleles could be retargeted into the readily differentiatable, feeder independent, E14 cell line 'E14-TG2 ' (kindly provided by Austin Smith). The differentiation capability of k.o. compared to wt cells can be studied by the application of various differentiation protocols. To follow the differentiation process in further detail, immunostainings with markers for the different stages of differentiation can be performed including the staining for terminal differentiation markers like -III-tubulin for neurons or artrial natriuretic factor (ANF) for cardiac muscle. In addition expression studies on the unverified potential target genes of Trx2 (otx2 and hoxb1) in Trx2-/- cells at different stages of differentiation would compliment our previous unpublished observations.

Since the use of the EYFP-tagged TRX2 protein failed to give faithful information about the cell biology of the protein as described in chapter R-2.2, the Trx2 specific antibody could serve an alternative tool to study TRX2 sub cellular localization. Even though this approach would not contain the merits of an in vivo localization system, still questions about a specific sub-nuclear distribution (comparable to the speckled localization pattern of CGBP and MLL), potential pattern changes after induction of a differentiation pathway or the attachment or detachment respectively of TRX2 to DNA during differentiation could be addressed.

D-8.2   Is Trx2 involved in chromatin regulation ?

Since indications from *Drosophila* suggest that Trx2 acts on target gene expression as a maintenance factor for active chromatin, the Trx2-/- ES cells also present unique experimental possibilities for detailed analyses of the chromatin aspect for mammalian

Trx2. The processing of a specific Trx2 antibody now allows to perform **Ch**romatin **I**mmuno **P**recipitation (ChIP) assays on the genomic locus of potential target genes to learn about the presence or absence of TRX2 at their regulatory regions. Those ChIP experiments could comprise the analysis of DNAse hypersensitivity sites in enhancer regions, the tracking of expression activity and the correlation of TRX2 presence with both of them. Since Trx has been identified to have HMT activity, studies on the methylation status of the target chromatin could be a valuable continuative experiment.

D-8.3 Further use of EYFP-Trx2 mice

EYFP homozygous adult survivors dealt with in chapter R2.4 could be used to further address the differences between the wt and the EYFP-tagged Trx2 allele. An interesting question could be whether the EYFP homozygous males are able to transmit the EYFP allele to the F1 generation through the male germline. By crossing them to heterozygous females we expect a mendelian rate of 50% hom and 50% het offspring. If the actual rate would be in this range it can be concluded that those males have probably adapted to the EYFP-Trx2 allele by some stably transmittable process.

EYFP homozygous females could serve as a model system to understand the molecular reasons for the defect in the development of female reproductive organs. A detailed analysis of the retarded ovaries of EYFP homozygous females concerning follicular development and granulosa cell differentiation is suggested.

The existence of mouse lines in our lab that carry other Trx2 alleles (Trx2 k.o. and TAP-Trx2) opened the possibility to potentially create a spectrum of hypomorphic combinations upon crossing of those available lines with the EYFP allele. Breedings between a female heterozygous for the Trx2 k.o. allele and a male homozygous for the EYFP allele results in 50% offspring that carry a combination of both Trx2 alleles and should be characterized by a fetal phenotype that is classified between those of both homozygous variants (lethality between E10.5 and birth). Study on those newly created phenotypes would allow further insight into Trx2 late function in development.

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Abbreviations

ATP	adenosine triphosphate
bp	base pairs
C-terminus	carboxy-terminus
dH2O	distilled water
DMT	DNA methyl transferase
DNA	desoxyribonucleic acid
dw	downstream
E10.5	embryonic day 10.5
ES	embryonic stem cell
et al.	and others
EYFP	enhanced yellow fluorescent protein
H	histone
HAT	histone acetyl transferase
HDAC	histone deacetylase
het	heterozygous (+/-)
HMT	histone methyl transferase
hom	homozygous (-/-)
ICM	inner cell mass
K	lysine
k.o.	knock-out
kb	kilobases
LacZ	galactosidase enzyme
min	minute/s
Mll	mammalian mixed lineage leukemia gene
MLL	mammalian mixed lineage leukemia protein
mRNA	messenger RNA
N-terminus/ C-terminus	amino-terminus/ carboxy-terminus
o/n	over-night
ORF	open reading frame
PAGE	Polyacrylamid gel electrophoresis
Pc-G	polycomb group of proteins
PEV	position effect variegation
PLB	protein loading buffer
RNA	ribonucleic acid
rRNA	ribosomal RNA
SAM	S-adenosylmethionine
sec	second/s
TAU	triton/Acid/Urea
Trx	fly trithorax gene
TRX	fly trithorax protein
Trx2	mammalian trithorax 2 gene
TRX2	mammalian trithorax 2 protein
trxG	trithorax group of proteins
up	upstream
wt	wildtype

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EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eidesstatt, dass ich die vorliegende Arbeit selbstständig verfaßt, und keine unzulaessigen oder nicht angegebenen Hilfsmittel benutzt habe.
Die Arbeit hat, weder in dieser noch in ähnlicher Form bereits einer anderen Prüfungsbehörde vorgelegen.

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