DNA Replication in Animal Systems
Lacking Thioredoxin Reductase I

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1. Introduction

Ribonucleotide reductase (RNR) activity is generally required to provide deoxyribonucleoside triphosphates (dNTPs or DNA-precursors) for DNA replication (Thelander and Reichard, 1979). This property has made both RNR and the pathways RNR depends upon important drug-targets. For example, the drug hydroxyurea is a specific inhibitor of RNR and has been used for many decades as an effective chemotherapeutic agent for certain cancers and viral diseases (Navarra and Preziosi, 1999; Wright et al., 1990; Yarbro, 1992). This chapter focuses on two critical pathways that lie upstream of RNR and are important for supporting RNR activity: namely, the glutathione (GSH) pathway and the thioredoxin (Trx) pathway. These pathways were first uncovered in bacterial systems roughly fifty years ago. In the ensuing half-century, the components and activities of these pathways have been intensely studied in bacterial, archaeabacterial, and eukaryotic systems, both in vivo and in vitro (Holmgren, 1977; Holmgren, 1989). The GSH and Trx pathways, themselves, are ubiquitous in biology, yet various components of the pathways exhibit activities and, in some cases, evolutionary histories, that are particular to animal systems. Classic descriptive and biochemical studies laid the groundwork for understanding these pathways in animals; however, only in recent years have genetic systems been established in which the physiological activities of these pathways could be tested (Arner, 2009; Holmgren and Lu, 2010; Holmgren and Sengupta, 2010). Here I will overview the Trx and GSH pathways and their contributions to DNA replication. Particular attention will be paid to recent revelations on the activities and properties of these systems in animals that differ from those in other biological systems. Some recent advances have come from the development of mouse models bearing targeted “conditional” alleles of the gene encoding thioredoxin reductase I (TrxR1, also called Txnrd1 or TR1), which can be disrupted in a cell type- or developmental stage-specific manner. Whereas these models are yielding some exciting insights into the Trx and GSH systems in embryonic development, stress responses, toxicology, cancer, and other processes (Bondareva et al., 2007; Branco et al., 2011; Carvalho et al., 2008; Jakupoglu et al., 2005; Mandal et al., 2010; Rogers et al., 2004; Suvorova et al., 2009; Tipple et al., 2007; Zhang and Lu, 2007), the current treatise will emphasize the interplay of these pathways in supporting DNA replication in animal systems. The enormity of the body of literature on the Trx, GSH, and RNR systems precludes an exhaustive review of these materials, and it is my intention to cover these subjects in only a cursory manner to set the backdrop for understanding these systems in the context of DNA replication in animals. The reader is directed to more
complete recent reviews on these subjects (Arner, 2009; Holmgren and Sengupta, 2010; Lu and Holmgren, 2009). I apologize in advance for my oversights and omissions related to the many important studies that have led to the current status of the field.

2. Thioredoxin reductase and glutathione reductase

Thioredoxin reductases (TrxRs) are enzymes that use electrons from NADPH + H\(^+\) to restore the “active” reduced state of oxidized Trx (Fig. 1). Similarly, glutathione reductases (Gsrs) are enzymes that use electrons from NADPH + H\(^+\) to convert oxidized glutathione disulfide (GSSG) into two molecules of reduced GSH (Arner and Holmgren, 2000; Holmgren, 1980; Holmgren, 2000). In both cases, electrons are typically exchanged as a “reductive currency” by altering the redox state of protein- or small molecule-sulfur residues. In combination, these two pathways provide reducing potential to countless reactions in cellular, subcellular and extracellular compartments, and constitute the predominant endogenous antioxidant system (Arner, 2009; Arner and Holmgren, 2006; Holmgren, 2000; Lillig and Holmgren, 2007; Nordberg and Arner, 2001). Trx and GSH serve as “electron-shuttles”, transporting this reducing potential to various enzymes and reactions. Both systems participate in homeostatic antioxidant activities, for example by providing electrons to either the GSH-dependent glutathione peroxidases (Gpxs) or the Trx-dependent peroxiredoxins (Prxs) that each contribute to detoxification of reactive oxygen species in cells.

Fig. 1. Cytosolic thioredoxin reductase cycle. Reducing potential arrives at TrxR1 in the form of NADPH and a proton. TrxR1 reduces disulfide-form Trx1 (oxidized) to the dithiol-form. Trx1 is a small protein that can transport this reducing potential to locations throughout the cell; however it does not enter or exchange reducing potential with mitochondria. Reduced Trx1 is a major protein-disulfide reductase and the immediate source of electrons for many enzymatic reactions, including RNR, Prxs, 3'-phosphoadenylylsulfate (PAPS) reductase, methionine sulfoxide reductases, and others. This results in oxidation of Trx1 to the disulfide form, which then cycles back through TrxR1 (Arner, 2009; Holmgren and Bjornstedt, 1995).
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(Arner and Holmgren, 2000; Berndt et al., 2007; Carmel-Harel and Storz, 2000; Holmgren, 2000). GSH can also reduce glutaredoxins (Grxs), which are small Trx-like proteins that further shuttle the electrons to various destinations, often but not always functioning analogous to Trxs (Fernandes and Holmgren, 2004; Holmgren, 1989; Holmgren, 2000; Holmgren et al., 2005; Meyer et al., 2009; Meyer et al., 2008). Although the Trx pathway and the GSH pathway are each most well known for somewhat different roles (Berndt et al., 2008; Carmel-Harel and Storz, 2000; Hayes and Pulford, 1995; Holmgren, 2000), there is enormous overlap between the two pathways and, for the most part, general physiological equivalence of the two pathways. Some evidence suggests the Trx pathway can respond to cellular damage by activating the cytoprotective Nrf2 stress-response pathway, which, in turn, induces expression of the genes encoding components of both the Trx and the GSH systems (Arner, 2009; Ishii and Yanagawa, 2007; Itoh et al., 1999; Nguyen et al., 2009; Suvorova et al., 2009). Generally, either the GSH or the Trx pathway can complement deficiencies in the other, resulting in a robust combined reductive system (Arner, 2009; Holmgren, 2000).

3. Activities and requirements of RNR

The role of RNR in replication is to provide dNTPs for synthesis of a duplicate genome by DNA polymerase during S phase of the cell cycle (Fig. 2). This pathway, in which DNA-precursors are made from endogenous RNA-precursors is termed the de novo biosynthesis pathway. Although salvage pathways can also provide a source of dNTPs using exogenous deoxyribonucleosides (Arner and Eriksson, 1995), sufficient exogenous sources of these substrates are rarely available. Thus, whereas salvage pathways might provide a sufficient source of DNA precursors for repair and perhaps for mitochondrial DNA replication, they generally will not support S phase genome replication (Iwasaki et al., 1997; Mathews and Song, 2007; Pontarin et al., 2007).

RNR functions by reductive conversion of generally abundant RNA precursors, in the form of ribonucleoside diphosphates (rNDPs), into deoxyribonucleoside diphosphates (dNDPs) (Holmgren et al., 1965; Reichard et al., 1961; Thelander and Reichard, 1979). The dNDPs are subsequently phosphorylated to the triphosphate state for use by DNA polymerase. In eukaryotes, although genomic replication is nuclear, RNR and dNTP biosynthesis occurs in the cytosol. dNTP pools in replicating cells are at a low steady-state concentration and are rapidly turned-over, indicating that the precursors are polymerized into DNA almost immediately upon their production (Rottgen and Rabes, 1989). Indeed, cellular DNA precursor pools, even during S phase, are typically only a small fraction of the concentration of RNA precursor pools (Rottgen and Rabes, 1989; Spyrou and Holmgren, 1996). Consistent with this, RNR shows tight product- and substrate-mediated allosteric regulation, in particular in response to local concentrations of dATP (Holmgren, 1981; Holmgren et al., 1965; Reichard et al., 2000). This feedback regulation is thought to be critical for maintaining replication fidelity and preserving genome-integrity. Thus, replication accuracy by DNA polymerase is optimal only within a narrow window of concentrations for each dNTP; treatments that skew normal dNTP pools have been shown to be either mutagenic or pro-apoptotic in different systems (Kunkel et al., 1982; Nicander and Reichard, 1983; Oliver et al., 1996).

Although three different classes of RNR enzymes are known to exist across the different biological kingdoms, all of these enzymes require a source of electrons and a metal co-factor,
and all proceed by a reaction that involves a free radical intermediate (Atkin et al., 1973; Holmgren and Sengupta, 2010; Thelander and Reichard, 1979). Classes II and III are found only in a restricted subset of anaerobic or otherwise specialized microbes. These enzymes were discussed in detail in a recent review (Holmgren and Sengupta, 2010) and will not be considered further here. The most common class, class I, is found in eukaryotes and in most aerobic prokaryotes (Torrents et al., 2006). These enzymes are composed of two subunits: the B1 and B2 proteins in bacteria, or the functionally similar R1 and R2 proteins (also called M1 and M2) in eukaryotes (Brown et al., 1969; Thelander and Reichard, 1979). The B1 or R1 protein possesses the catalytic site for reduction of all four rNDPs, whereas the B2 or R2 subunit contains the protein-tyrosyl radical (Akerblom et al., 1981; Avval and Holmgren, 2009). Every cycle of nucleotide reduction results in generation of disulfide in the C-terminus of the B1 or R1 subunit, which must be reduced to a dithiol for the next reductive cycle (Avval and Holmgren, 2009).

Fig. 2. Sources of DNA precursors. RNR is the key player on the de novo dNTP biosynthesis pathway, in which rNDPs are converted to dNDPs in eukaryotes and most prokaryotes. In addition to rNDPs, RNR requires a source of electrons as reducing potential (green arrow, see text). Arrow weight is diagrammatic of relative flux or activity of each step. The drug hydroxyurea (HU) scavenges the tyrosyl protein-free radical in RNR, thereby blocking RNR activity (red). Below is the salvage pathway, in which exogenous deoxyribonucleosides are assimilated by outer membrane transporters and kinased to form dNTPs. BrdU (blue) is incorporated via the salvage pathway, which, although it cannot generally support S phase replication (see text), does contribute sufficient DNA precursors to genome replication that BrdU will label all S phase (replicating) cells (Arner and Eriksson, 1995; Thelander and Reichard, 1979; Yarbro, 1992).
Mammalian cells have two distinct RNR enzymes that both share a common catalytic R1 subunit, but differ in their second subunit, with one isoform being S phase-specific and containing R2 protein and the other being expressed throughout the cell cycle as well as in post-replicative cells and having a distinct protein, p53R2, as the second subunit (Avval and Holmgren, 2009; Holmgren and Sengupta, 2010; Pontarin et al., 2007). The R1/R2 protein drives S phase genome replication; the R1/p53R2 protein is thought to play a major role in providing dNTPs for mitochondrial DNA replication and repair (Pontarin et al., 2007).

In addition to an obvious requirement for rNDPs, RNR requires a source of reducing potential or, more specifically, electrons, to restore the dithiol state of the B1 or R1 C-terminal disulfide after each catalytic cycle (Fig. 3) (Holmgren and Sengupta, 2010). The common distal source of this reducing potential is NADPH. In eubacteria, electrons can flow to RNR from NADPH by either a TrxR- or a Gsr-dependent route (Fernandes and Holmgren, 2004; Gleason and Holmgren, 1988; Holmgren, 1976; Holmgren, 1981; Holmgren, 1989; Laurent et al., 1964; Lillig and Holmgren, 2007). In the TrxR-dependent route, TrxR uses electrons from NADPH + H+ to reduce oxidized (disulfide) Trx to the reduced (dithiol) form, while generating NADP+ (Arner, 2009; Arner and Holmgren, 2000). Reduced Txn, then, can directly restore the active reduced state of RNR (Avval and Holmgren, 2009; Laurent et al., 1964). In the Gsr-dependent route, electrons extracted from NADPH + H+ are used by Gsr to reduce oxidized di-glutathione (GSSG) to the reduced monomeric state (GSH), again yielding NADP+ (Fernandes and Holmgren, 2004; Holmgren, 2000). Whereas GSH has numerous activities in cells (Fernandes and Holmgren, 2004), one of these is to restore oxidized (disulfide) Grxs to the reduced state (dithiol-Grx), which like reduced Trx, can restore the reduced active state of RNR (Avval and Holmgren, 2009; Holmgren, 1976; Holmgren, 1977; Holmgren, 1978; Holmgren, 1979; Luthman et al., 1979) (Fig. 3).

Fig. 3. Sources of electrons for RNR. Either the Trx cycle (blue) or the GSH cycle (green) can support reduction of ribonucleotides by RNR. The Trx cycle is summarized in Fig. 2. In the GSH cycle, Gsr uses reducing potential from NADPH to reduce one mole of oxidized di-glutathione (GSSG) to 2 moles of reduced glutathione (GSH). GSH has many roles in cells, one of which is to restore oxidized Grxs to the reduced state (dithiol-Grx), which like reduced Trx, can restore the reduced active state of RNR (Avval and Holmgren, 2009; Holmgren, 1976; Holmgren, 1977; Holmgren, 1978; Holmgren, 1979; Luthman et al., 1979) (Fig. 3).

4. Contributions of the GSH and Trx pathways in supporting RNR

Although growth differences have not been reported between E. coli having only a GSH- or only a Trx-pathway (Holmgren, 1977; Holmgren, 1979), catalytically Grx is the most efficient
electron donor for the reaction (Gon et al., 2006). By contrast, yeast and plants deficient in TrxRs show slow-growth phenotypes (Koc et al., 2006; Reichheld et al., 2007; Sweat and Wolpert, 2007), suggesting Gsr-dependent routes are poor at supporting DNA replication in these eukaryotes. It was anticipated that all eukaryotic systems might depend primarily on the Trx system to supply electrons to RNR for DNA replication. Consistent with this, mice homozygous for a spontaneous null mutation of the gsr gene showed no defects in growth or DNA replication (Rogers et al., 2004), and mice zygotically homozygous-null for either the txn1 gene encoding cytosolic Trx1 (Matsui et al., 1996) or the txnrd1 gene encoding cytosolic TrxR1 (Bondareva et al., 2007; Jakupoglu et al., 2005), are both embryonic-lethal. From these findings, it was inferred that, in mice under normal conditions, the GSH pathway may be superfluous for replication, yet the cytosolic Trx pathway is critical (Jakupoglu et al., 2005). However, since none of the studies on components of the cytosolic Trx systems directly showed that these mutations blocked DNA replication, per se, alternative explanations for embryonic lethality were not ruled out. Indeed, in reporting our study, we argued that the degree of cell proliferation seen prior to embryonic loss in TrxR1-deficient embryos was inconsistent with a block to proliferation, and instead suggested that the pre-resorption phenotype of TrxR1-deficient embryos was more consistent with an embryonic patterning defect (Bondareva et al., 2007)(see below).

5. Evolution of the TrxR protein families

GSH- and Trx-pathways are each ubiquitous in biology (Fig. 4). With the advent of organellar compartmentalization in early eukaryotes, it likely became important for cells to ensure an adequate level of activity for each pathway both in the cytosol as well as within the often relatively impervious confines of the organelles. For most components of these pathways in most eukaryotic systems, such as Grxs, Trxs, and TrxRs, separate genes arose by gene duplication that evolved to specialize, albeit to varying extents, in production of either cytosolic or mitochondrial isoforms of these enzymes (Gleason and Holmgren, 1981; Meyer et al., 2009; Meyer et al., 2008; Novoselov and Gladyshev, 2003; Sandalova et al., 2001; Taskov et al., 2005; Williams et al., 2000). For other components of these pathways, however, such as Gsr, cytosolic and mitochondrial functions are generally accomplished by expressing both cytosol- and mitochondria-targeted versions of the protein from a single gene. In an extreme example, the parasitic tapeworm Echinococcus has a single gene that issues the enzyme responsible for reduction of both Txr and GSSG in both the cytosol and the mitochondria (Bonilla et al., 2008) (see below).

Despite the ubiquity of these systems in the living world, TrxR enzymes underwent a striking evolutionary transition that sets it apart from other components in these systems (Zhong et al., 1998). Thus, whereas all Gsrs are homologous, all Grxs are homologous, and all Trxs are homologous, TrxRs are diphyletic, being represented by two distinct protein families (Arner, 2009; Arner and Holmgren, 2000; Sandalova et al., 2001). The more ancient family, here called the “E. coli-type TrxRs” for the species it was first described from (Moore et al., 1964), is found in all eu-bacteria, archaea, fungi, most protists, and most plants and algae (Fig. 4., shaded light blue). Metazoan animals universally share a distinct family of TrxR proteins (“metazoan-TrxRs”; shaded red in Fig. 4) (Arner, 2009; Arscott et al., 1997; Eckenroth et al., 2006; Novoselov and Gladyshev, 2003; Williams et al., 2000; Zhong et al., 1998).
Fig. 4. Distribution of TrxR families in living kingdoms. E. coli-type TrxRs (blue) are found in most extant life forms, with the exception of metazoans (red), one green algae, and a few parasitic protists (red dots), which have metazoan-type TrxRs (Novoselov and Gladyshev, 2003). Thus, the metazoan-type TrxRs appeared before separation of ancestral metazoans onto a distinct lineage and was retained in a subset of plants and algae and in a subset of protists (Novoselov and Gladyshev, 2003) (fine red lines). See text for more details.

Evolution of the metazoan TrxR appears to have been brought about by extension of the C-terminal protein coding sequences of the gsh gene, leading to acquisition of the new C-terminal active site (Novoselov and Gladyshev, 2003). In this enzyme, the C-terminal active site, which directly reduces oxidized Trx, generally but not always contains the atypical 21st amino acid selenocysteine (Sec) translationally inserted in the penultimate position (Arner, 2009; Hondal and Ruggles, 2010; Lu and Holmgren, 2009). This overall design gives the impression of an enzyme that was built by “retrofitting” a new C-terminal active site onto an existing NADPH-dependent reductase (Schmidt and Davies, 2007), either by classical exon shuffling (Margulies and McCluskey, 1985) or by mutation of the stop codon and translational read-through into previously 3’-untranslated sequences (Novoselov and Gladyshev, 2003).

It is unclear why an ancestor to all extant metazoans eventually discarded its E. coli-type TrxRs, which presumably functioned adequately in its ancestors and continues to do so for nearly all contemporary non-metazoans within all biological kingdoms (Fig. 4). From the perspective of the nominal reaction catalyzed by TrxRs in either E. coli or mammalian systems, neither the distal source of electrons (NADPH) nor the substrate (oxidized Trx) differs. However, the E. coli and metazoan enzymes are not equivalent, and this evolutionary enzyme-exchange was unlikely to be selectively “neutral”. Key differences between the two enzyme families are that the E. coli-type enzymes are smaller (~35 kDa) and highly specific for reduction of oxidized Trx, whereas metazoan TrxRs are larger proteins.
(~55 kDa) that evolved from Gsr and are capable of reducing a broad range of substrates (Arner, 2009; Arscott et al., 1997; Holmgren and Bjornstedt, 1995; Lu and Holmgren, 2009; Williams et al., 2000). Although they evolved from Gsr and have a broad substrate specificity, the classical mammalian TrxR1 and TrxR2 enzymes do not reduce GSSG (Sun et al., 2001). However, the testis-specific mammalian TrxR3 protein, also called thioredoxin-glutathione reductase or TGR, as well as the Sec-lacking TrxR protein in Drosophila, does reduce GSSG (Gromer et al., 2003; Johansson et al., 2006; Sun et al., 2001; Sun et al., 2005), and in some lower metazoans such as Echinococcus, a single gene, within the metazoan TrxR family and containing Sec in its C-terminal active site, encodes all known Trx- and GSSG-reductase activities (Bonilla et al., 2008). One might hypothesize that, by exchanging the ancestral E. coli-type TrxR enzymes for the metazoan version, the evolutionary capacity of the lineage might have been potentiated; however since both enzymes will effectively reduce Trx, this model suggests the evolutionary advantage of the new enzyme for metazoans is related to other activities that differ between these enzyme types (Arner and Holmgren, 2000; Arner et al., 1996; Lothrop et al., 2009). Alternatively, one might imagine that, if an ancestral metazoan evolved a TGR enzyme that could replace both the ancestral TrxR and Gsr activities, as the Echinococcus version does, then perhaps the ancestral E. coli-type TrxR and the ancestral Gsr were simply and irrevocably lost as being redundant with the new bi-functional TGR enzyme, as seen in Echinococcus. Subsequent specialization could have led to a gene-duplication of TGR and loss of the C-terminal TrxR-specific domain in one of the duplicates, essentially reverting one copy of the gene to a classical Gsr protein that is still recognized as homologous to the ancestral version.

Interestingly, a few unicellular eukaryotes do contain “metazoan-type” TrxR enzymes (Novoselov and Gladyshev, 2003)(Fig. 4, red “dots” at top of “Plant and algae” and “Protist” branches of the tree). These include both a small number of protozoan parasites and a single known green alga (Holmgren and Lu, 2010; Novoselov and Gladyshev, 2003). In at least one case (the photosynthetic alga Chlamydomonas), both E. coli- type and metazoan-type TrxRs are found in the same genome (Novoselov and Gladyshev, 2003). Thus, there is a precedent for co-existence of both enzyme types in a single genome. One model to explain this odd distribution of the metazoan-type TrxR would be that these few non-metazoan species acquired the metazoan-type TrxR proteins by lateral gene transfer. Indeed, this hypothesis was raised previously when this isoform was discovered in members of genus Plasmodium, the malaria-causing parasites, and in other related protozoan parasites (Hirt et al., 2002; Rahlfs et al., 2002; Williams et al., 2000). Based both on the oddity of non-metazoans having this isoform and the intimate intracellular interaction between these intracellular protozoan parasites and both their vertebrate and invertebrate metazoan hosts, lateral gene transfer from host to parasite seemed a plausible model for the appearance of this unexpected isoform outside of metazoans. However, the subsequent discovery of a metazoan-type TrxR1 in Chlamydomonas, along with the absence of other signatures of lateral gene transfer between metazoans and the few single-cell eukaryotes that have this isoform, suggest lateral gene transfer is, in this case, an unlikely model (Novoselov and Gladyshev, 2003). Instead, the most parsimonious model of ancestry of the metazoan-type TrxRs is that the protein evolved only once by C-terminal extension of a copy of the gsr gene in a pre-metazoan ancestor; although lineages bearing this enzyme persisted to modern times in all metazoans as well as in a very small subset of algae and parasitic protozoa (Fig. 4, fine red lines) (Novoselov and Gladyshev, 2003).
6. Requirements of disulfide reductases for replication in rodent models

Early studies showed that some mammalian Grxs could reduce mammalian RNR in vitro (Luthman et al., 1979; Luthman and Holmgren, 1982), and that Trx generally did not colocalize to cells expressing the S phase-specific R1 subunit of RNR in rat tissues, suggesting that Trx is not the major physiological electron donor for RNR (Hansson et al., 1986). However experimentally, animal systems deficient in the Trx pathway were slow to appear. RNAi “knock-down” studies on mammalian cell cultures suggested replication was not impaired by ablation of TrxR1 (Yoo et al., 2006; Yoo et al., 2007); however questions of residual pre-formed TrxR1 protein in these systems lingered. The first studies on mouse knock-out models having homozygous zygotic disruption of the txnrd1 gene, which encodes TrxR1, showed evidence of active and extensive proliferation prior to embryonic lethality (Bondareva et al., 2007; Jakupoglu et al., 2005). Because TrxR1-deficient embryos accumulated several thousand cells, it was unlikely that residual maternal TrxR1 had driven the replication cycles. More recently, by using conditional disruption of the txnrd1 gene in mouse livers, my group has been able to provide a more detailed examination of the roles of TrxR1 for replication in animal cells (Rollins et al., 2010; Suvorova et al., 2009). During liver development and regeneration, normal mice and mice having TrxR1-deficient hepatocytes exhibit similar liver growth rates and similar levels of proliferative, S, and M phase hepatocytes. Regenerative thymidine incorporation is similar in normal and TrxR1-deficient livers, further indicating that DNA synthesis is unaffected (Rollins et al., 2010). The use of genetic chimeras in which a fluorescently marked subset of hepatocytes was TrxR1-deficient while others were not, revealed that the multigenerational contributions of both normal and Txnrd1-deficient hepatocytes to development and to liver regeneration were indistinguishable (Rollins et al., 2010). Thus, TrxR1 is truly superfluous for DNA replication and RNR activity in otherwise normal mouse hepatocytes.

Questions remain as to whether TrxR1-independent replication will prove to be a general phenomenon of mammalian cells or a peculiarity of hepatocytes. Both primary papers on mouse embryos lacking TrxR1 reported that it proved impossible to establish cultures of primary fibroblasts from mutant embryos; however it was not established whether this failure resulted from a block to replication or some other defect (Bondareva et al., 2007; Jakupoglu et al., 2005). As time passes, more and more cell types are being found to replicate in the absence of TrxR1. Recently, it was shown that lymphomas lacking TrxR1 can initiate and progress normally in mice (Mandal et al., 2010), adding another cell type to the list of cell types that can replicate in the absence of TrxR1. Also, in investigations using a fluorescent marker-tagged system in which both copies of the TrxR1 gene were disrupted in an arbitrary population of all cell types in fetal mice, my group assessed which, if any, cell types failed to contribute to the adult mouse two months later. No cell types could be identified that did not still contribute to the adult mouse under these conditions (CM Weisend and EE Schmidt, unpublished). Thus, whereas we cannot exclude the possibility that there could be some rare cell types in mice in which replication is critically dependent on TrxR1, our efforts to date have failed to pinpoint any such cell types.

Recently, a study was reported on disruption of TrxR1 in another metazoan, the nematode C. elegans (Stenvall et al., 2011). C. elegans has only a single TrxR protein, and this is the only Sec-containing protein in the worm genome (Stenvall et al., 2011). Surprisingly, although worms lacking TrxR1 show a molting defect due to an inability to reduce protein disulfides in the old cuticle to allow its removal, but like mouse livers lacking TrxR1 (Rollins et al., 2010; Suvorova
et al., 2009), they show no evidence of oxidative stress or replicative insufficiency (Stenvall et al., 2011). Like in mice (Rollins et al., 2010), the source of electrons for RNR in worms remains unclear and will likely be an important subject for future investigation.

7. Alternative pathways of supplying electrons to RNR

Mammalian genomes contain three different genes encoding thioredoxin reductases. The *txnrd1* gene encodes TrxR1, the cytosolic enzyme; *txnrd2* encodes TrxR2 (also called Txnrd2 or TR3), the mitochondrial enzyme; and *txnrd3* encodes TrxR3 (also called Txnrd3, TGR, or TR2), a testis-specific cytosolic enzyme that can reduce either Trx1 or GSSG (Arner, 2009; Gerashchenko et al., 2010; Su et al., 2005). In most normal rodent tissues with the exception of testis, TrxR1 mRNA is about five-fold more abundant than TrxR2 mRNA, and TrxR3 mRNA is undetectable (Jurado et al., 2003). Previously, we have shown that neither TrxR2 mRNA nor TrxR3 mRNA are induced in TrxR1-deficient embryos (Bondareva et al., 2007) or livers (Suvorova et al., 2009), indicating that there is not an induction of the genes encoding either of the other known TrxRs that might compensate for ablation of TrxR1. However, it remained possible that normal levels of TrxR2 protein, though perhaps lower than normal levels of TrxR1 protein (see above), might be sufficient to compensate for loss of TrxR1 without an associated increase in mRNA levels. It is well established that the cytosolic and mitochondrial TrxR enzymes in *Arabidopsis* are cross-complementary. Thus, disruption of the gene encoding either of these *E. coli*-type TrxRs is compensated by a partial re-distribution of product from the other gene into the deficient sub-cellular compartment (Meyer et al., 2008; Reichheld et al., 2007; Sweat and Wolpert, 2007). To date, there is no *in vivo* evidence of similar cross-complementation occurring in mammalian systems. Nevertheless, several ESTs issued from the *txnrd2* gene but lacking the N-terminal mitochondrial transit signal have been reported from mammalian systems, which suggests the possibility that the *txnrd2* gene could yield cytosolic isoforms of TrxR2 (Turanov et al., 2006). Closer examination of this possibility will be important for determining whether cytosolic isoforms of TrxR2 participate in replication or homeostatic maintenance of mammalian cells lacking TrxR1.

Another system that might be compensating for loss of TrxR1 and supporting RNR activity and DNA replication in TrxR1-deficient hepatocytes is the GSH system. Neither livers nor embryos lacking TrxR1 exhibit compensatory induction of Gsr; however they do exhibit induction of mRNA encoding the modifier subunit of glutamate-cysteine ligase (Gclm), the rate limiting enzyme in GSH biosynthesis (Bondareva et al., 2007; Suvorova et al., 2009). A recent study showed that the GSH pathway, reconstituted entirely from recombinant or purified components (Gsr, GSH, and Grx), can effectively transfer electrons from NADPH to RNR and drive reduction of ribonucleoside diphosphates *in vitro* (Avval and Holmgren, 2009). In combination with the classical study showing that S-phase cells in normal rat tissues did not tend to exhibit Txr immunostaining (Hansson et al., 1986), there is a good possibility that the GSH system is supporting RNR activity in TrxR1-deficient hepatocytes (Holmgren and Sengupta, 2010); however other possibilities still exist, and all of these will need to be tested *in vivo*.

8. Considerations beyond supplying electrons to RNR

Decades of elegant studies on microbial and other non-metazoan systems revealed that, typically, if inhibition of disulfide reductases disrupted the supply of electrons to RNR,
growth was abated; if not, growth was overtly normal (Gleason and Holmgren, 1988; Holmgren, 1985). In some cases, disruption of the Trx system was reported to yield intermediate growth phenotypes in non-metazoan eukaryotic systems (Koc et al., 2006; Meyer et al., 2009; Meyer et al., 2008; Reichheld et al., 2007; Sweat and Wolpert, 2007), apparently due to compromised output by RNR (Koc et al., 2006). Some studies on microbial models have shown that disruption of the Trx system can independently cause a disruption in homeostatic redox control or stress responses (Arner and Holmgren, 2000; Carmel-Harel et al., 2001; Carmel-Harel and Storz, 2000; Holmgren, 2000). Also, in mammalian systems, an intimate connection between intracellular signaling by the platelet-derived growth factor receptor (PDGFR), the T cell receptor, the epidermal growth factor receptor (EGFR), the tumor necrosis factor-α receptor (TNFAR), and likely other related phosphotyrosine receptors and Prxs has been established (Baé et al., 1997; Choi et al., 2005; Devadas et al., 2002; Kang et al., 1998; Rhee, 1999; Rhee et al., 2000), thereby indirectly linking the Trx pathway with physiological growth factor signaling. Thus, it is important to bear in mind that disruptions of cell physiology in response to perturbations in the Trx or GSH pathways, in particular in whole animals or other complex systems, is not necessarily indicative of a replicative block, and these distinctions can sometimes be difficult to tease apart experimentally.

A case in point is the as yet mechanistically uncertain causes of embryonic lethality in TrxR1-deficient mice (Bondareva et al., 2007; Jakupoglu et al., 2005). My group and that of Dr. Markus Conrad each generated independent and strategically distinct conditional-mutant alleles of the *txnrd1* gene that, in the zygotically homozygous-mutant state, result in embryonic lethality (Bondareva et al., 2007; Jakupoglu et al., 2005). In each case, the homozygous-mutant zygote proliferates to yield thousands of TrxR1-deficient cells prior to embryonic failure. Subsequently, using regulated disruption of these conditional alleles, mouse cells homozygous null for either allele have been shown to replicate vigorously (Mandal et al., 2010; Rollins et al., 2010), suggesting proliferation is normal in TrxR1-deficient cells. Why, then, do the mutant embryos die?

Although the reported details of embryonic progression differ between the two alleles, the system developed in my lab, with which I am most familiar, shows a phenotype that I believe lends clues to the answer. Despite the mutant embryos surviving and proliferating to embryonic day 8.5 (E8.5), the embryos become phenotypically abnormal much earlier (Bondareva et al., 2007). During post-blastocyst development, we detect no evidence of node formation, no development of primitive streak, and no differentiation of mesoderm (Bondareva et al., 2007). As a result, no body axis forms, normal patterning is not established, and the TrxR1-deficient embryonic cells proliferate as a disorganized mass of primitive endoderm and ectoderm until, by E8.5, they have likely exceeded the volumetric constraints for survival without a functional (mesoderm-derived) cardiovascular and hematopoietic system. At this point, the embryo likely becomes necrotic and is simply resorbed by the mother (Bondareva et al., 2007). This phenotype is consistent with failure of early morphogen-signaling events. To date, we have not entirely ruled-out a possible proliferative defect in some unidentified early embryonic cell type as underlying embryonic lethality. Indeed, the recently reported metabolic eccentricities of mouse embryonic stem cells for replication (Wang et al., 2009) might suggest one rare but critical cell type that needs TrxR1 activity for full replicative potential, and whose perturbation might disrupt formation of node, primitive streak, and mesoderm. Further investigations will be required to test this possibility. However, the proliferative characteristics of the TrxR1-deficient
mouse cells that have been studied to date disfavor this model (Mandal et al., 2010; Rollins et al., 2010). Conversely, the known interdependence of growth factor signaling on the Trx pathway (Choi et al., 2005; Rhee et al., 2005) is consistent with this embryonic phenotype. It is anticipated that these possibilities will be resolved in the very near future.

9. Summary and implications

Genome replication in most living systems is critically dependent on the activity of RNR. DNA synthesis is fairly rare in healthy adult mammals, being restricted to repair, mitochondrial renewal, and genome replication for a small subset of cells, including immune cells, germ cells, cells of the hair follicles, some intestinal epithelial cells, and a few others. However, certain diseases, such as cancers and some viral diseases, are critically dependent on DNA replication, and therefore are frequently combated with drugs that block this process. The key dependence of replication on RNR has made RNR an important drug-target for cancer and viral chemotherapies. Indeed, the classic chemotherapeutic drug HU directly inhibits RNR (Hatse et al., 1999; Lori and Lisziewicz, 1998; Newton, 2007; Romanelli et al., 1999; Szekeres et al., 1997; Yarbro, 1992), and various newer chemotherapeutics function similarly (Mayhew et al., 2002; Mayhew et al., 2005; Smart, 1995; Szekeres et al., 1994; Tsimberidou et al., 2002). Unfortunately, as with many chemotherapeutics, intra-host evolution of drug resistance by the diseased tissue is a common problem (Akerblom et al., 1981; Balzarini, 2000; Wright et al., 1990). The absolute dependency of RNR on a suitable electron-donor system has long suggested alternative mechanisms for blocking RNR activity, in particular in cases of HU resistance. Drugs that target either the Trx pathway, such as aurothioglucose, or the GSH pathway, such as buthionine sulfoxamine (BSO) have been developed and are well tolerated (Arner and Holmgren, 2000; Arner and Holmgren, 2006; Griffith and Meister, 1985; Lu et al., 2007; Williamson et al., 1982). Recent whole animal genetic studies reviewed here, however, suggest that in many if not all mammalian cells, either the GSH or the Trx pathway alone might be robust at supporting S phase RNR activity in the absence of the other. Thus, chemotherapeutic approaches to blocking DNA replication through disruption of disulfide reductase pathways will need to be cognizant of the potentially complete functional redundancy of the GSH and Trx pathways in animal systems, and sensitive to the various other physiological roles these pathways play in normal homeostatic and stress-response pathways.

10. References


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The study of DNA advanced human knowledge in a way comparable to the major theories in physics, surpassed only by discoveries such as fire or the number zero. However, it also created conceptual shortcuts, beliefs and misunderstandings that obscure the natural phenomena, hindering its better understanding. The deep conviction that no human knowledge is perfect, but only perfectible, should function as a fair safeguard against scientific dogmatism and enable open discussion. With this aim, this book will offer to its readers 30 chapters on current trends in the field of DNA replication. As several contributions in this book show, the study of DNA will continue for a while to be a leading front of scientific activities.

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