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Face off against ROS: *Tcof1*/Treacle safeguards neuroepithelial cells and progenitor neural crest cells from oxidative stress during craniofacial development

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Abstract

One-third of all congenital birth defects affect the head and face, and most craniofacial anomalies are considered to arise through defects in the development of cranial neural crest cells. Cranial neural crest cells give rise to the majority of craniofacial bones, cartilages and connective tissues. Therefore understanding the events that control normal cranial neural crest and subsequent craniofacial development is important for elucidating the pathogenetic mechanisms of craniofacial anomalies and for the exploring potential therapeutic avenues for their prevention. Treacher Collins syndrome (TCS) is a congenital disorder characterized by severe craniofacial anomalies. An animal model of TCS, generated through mutation of *Tcof1*, the mouse (*Mus musculus*) homologue of the gene primarily mutated in association with TCS in humans, has recently revealed significant insights into the pathogenesis of TCS. Apoptotic elimination of neuroepithelial cells including neural crest cells is the primary cause of craniofacial defects in *Tcof1* mutant embryos. However our understanding of the mechanisms that induce tissue-specific apoptosis remains incomplete. In this review, we describe recent advances in our understanding of the pathogenesis TCS. Furthermore, we discuss the role of *Tcof1* in normal embryonic development, the correlation between genetic and environmental factors on the severity of craniofacial abnormalities, and the prospect for prenatal prevention of craniofacial anomalies.

Keywords

craniofacial; DNA damage response/repair; neural crest cells; ROS; *Tcof1*

Introduction

Human faces exhibit an amazing diversity in size, shape and individual features. This diversity is derived from the complexity of craniofacial morphogenesis during early

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embryonic development. Central to proper craniofacial development is an important population of cells called cranial neural crest cells. Cranial neural crest cells comprise a multipotent, stem and progenitor cell population that forms most of the craniofacial bones, cartilages and connective tissues (Le Douarin & Kalcheim, 1999, Trainor, 2013). Consequently, defects in the formation, migration and/or differentiation of neural crest cells are thought to underlie many craniofacial malformations (Walker & Trainor, 2006, Crane & Trainor, 2006). Identifying the phase of neural crest cell development that is disrupted in association with a particular condition is very important for the understanding the pathogenesis of individual craniofacial abnormalities and for developing potential therapies. Conversely, analyzing craniofacial abnormalities can lead to the discovery of novel genes and/or mechanisms important for normal neural crest cell development, craniofacial morphogenesis and patterning.

Many congenital craniofacial abnormalities exhibit phenotypic variability without any correlation between genotype and phenotype. Environmental factors, such as pre-natal nutrition, and drug or chemical exposure are known to contribute to the severity of craniofacial abnormalities. However, how environmental and genetic factors interact and affect craniofacial development remains unclear. Animal models are a powerful tool for understanding the mechanisms that govern normal craniofacial development, for illustrating the pathogenesis of craniofacial abnormalities and uncovering the molecular mechanisms underlying the variability of phenotypic severity caused by environmental factors.

Here we initially summarize our knowledge of the multiple cellular functions of Tcof1, with an emphasis on the newly identified oxidative stress/DNA damage role during embryogenesis. Then we describe recent advances in our understanding of the pathogenesis of TCS, the effect of genetic and environmental factors on the severity of craniofacial anomalies and the prospect for prenatal prevention of TCS. Finally, we also discuss the role of endogenous reactive oxygen species on embryonic development.

What is Treacher Collins syndrome?

Craniofacial abnormalities account for approximately one-third of all congenital birth defects. Treacher Collins syndrome (TCS, OMIM number 1545000) is a severe congenital craniofacial disorder, and occurs with an incidence of 1 in 50,000 live births. Characteristic abnormalities associated with TCS include hypoplasia of jawbones and cheekbones, downward slanting of eyes, cleft palate and deformity of the external ears. Other clinical features of TCS include conductive hearing loss (Poswillo, 1975, Phelps *et al.*, 1981, Trainor *et al.*, 2009) and brain defects such as microcephaly and cognitive delay (Milligan *et al.*, 1994, Cohen *et al.*, 1995, Teber *et al.*, 2004). The gene primarily mutated in association with TCS is *TCOF1*, which is located on chromosome 5 (Treacher Collins Syndrome Collaborative Group, 1996). Over 200 family specific mutations have been identified in *TCOF1* gene and these include deletions, insertions, splicing, missense and nonsense mutations. The typical effect of these mutations is to introduce a premature termination codon which induces nonsense-mediated mRNA degradation and haploinsufficiency of *TCOF1*. *TCOF1* encodes a 144kDa protein called Treacle (Fig. 1A). Treacle contains a putative nuclear export signal at its N-terminus, as well as nuclear and nucleolar import

signals at its C-terminus, suggestive of dynamic localization (Winokur & Shiang, 1998). A LisH motif, which is typically associated with dimerization, microtubule binding, protein half-life and localization is also located at N-terminus (Wise *et al.*, 1997, Dixon *et al.*, 1997a). The most recognizable motifs in Treacle are the multiple casein kinase 2 (CK2)/ protein kinase C (PKC) phosphorylation sites in the central region of the protein (Isaac *et al.*, 2000). However, the importance of LisH motif and multiple CK2/PKC phosphorylation sites with respect to Treacle function remains unclear.

Although penetrance of the TCS-causing mutations is very high, the severity of the TCS phenotype varies between families and within families (Dixon *et al.*, 1994, Marres *et al.*, 1995). The most severe cases can result in perinatal death due to a compromised airway (Edwards *et al.*, 1996). However, some individuals are so mildly affected as to be indistinguishable from non-carriers of TCS-causing mutations. TCS associated mutations may therefore be carried more frequently than 1 in 50,000. Although over 200 family specific mutations have been identified, there is no clear evidence for a genotype-phenotype correlation (Gladwin *et al.*, 1996, Edwards *et al.*, 1997, Splendore *et al.*, 2000). Genetic background, environmental factors and/or stochastic events could all contribute to the clinical variability of TCS.

Tcof1 is expressed broadly throughout the embryo during all stages of development with elevated levels in the neuroepithelium and neural crest between E8.5–10.5 (Dixon *et al.*, 2006, Dixon *et al.*, 1997b). To understand the mechanisms underlying the pathogenesis of TCS, a mouse model of TCS was generated (Dixon and Dixon 2004). *Tcof1* homozygous mutants ($Tcof1^{-/-}$) exhibit embryonic lethality around E5.0, while haploinsufficient mutant ($Tcof1^{+/-}$) embryos mimic the characteristic features of TCS including hypoplasia of the maxillary and nasal bones (Dixon *et al.*, 2000). Importantly, $Tcof1^{+/-}$ embryos also mimic the intra- and inter-familial variability in craniofacial abnormalities observed in human patients, particularly with respect to the frontonasal hypoplasia which varies within litters (Dixon & Dixon, 2004). Furthermore, while, $Tcof1^{+/-}$ mice on mixed DBA;C57BL/6 background exhibit severe craniofacial defects reminiscent of TCS, and die within 24 hours of birth due to respiratory failure, in contrast, the majority of $Tcof1^{+/-}$ mice on a pure DBA background look normal, having only subtle facial and brain defects. Moreover, these mice are postnatal viable and fertile. Thus, the *Tcof1* knockout mouse is recognized as a good model for studying the pathogenesis of TCS.

Tcof1 is required for the maintenance of neural progenitor cells

Microcephaly is a congenital birth defect in which the cerebral cortex is considerably smaller than normal. Human genetic studies have identified links between mutations in genes associated with the process of mitosis and microcephaly. The majority of autosomal recessive primary microcephaly (MCHP) genes identified to date, including *ASPM, CDK5RAP2, CENPJ* and *NDE1*, encode centrosomal proteins. Mouse model studies have revealed that these genes are essential for the mitotic progression of neural progenitor cells and consequent proper neurogenesis (Fish *et al.*, 2006, Bond *et al.*, 2005, Feng & Walsh, 2004).

At the beginning of mitosis, the nucleolus disassembles in association with nuclear envelope breakdown, resulting in the release of proteins within the nucleolus and the nucleus into cytoplasm. Although, Treacle localizes to the nucleolus during interphase (Fig. 1B, Interphase)(Marsh *et al.*, 1998), it disperses into the cytoplasm and re-localizes at the centrosomes and kinetochores during mitosis (Fig. 1B; Mitosis)(Sakai *et al.*, 2012). This implies a mitotic function for Treacle, and consistent with this idea, *TCOF1* knockdown delays mitotic progression in HeLa cells due to impairment of chromosome alignment along the metaphase plate. Biochemical analyses revealed direct binding between Treacle and Polo-like kinase 1 (Plk1), a known master regulator of mitosis. Knockdown of *TCOF1* causes mis-localization of Plk1 and abnormal chromosome alignment in metaphase cells, illustrating the cooperation between these proteins during mitotic progression. Since *Tcof1*$^{+/-}$ mice on a pure DBA background exhibit smaller brains without craniofacial defects, the correlation between mitotic dysfunction and microcephaly was analyzed using this strain (Sakai *et al.*, 2012). BrdU-IdU labeling and immunostaining for the mitotic cell marker, phosphorylated histoneH3 revealed delays in the mitotic progression of neural progenitor cells in *Tcof1*$^{+/-}$ embryos. Furthermore, loss of *Tcof1* lead to randomization of the division plane of neural progenitor cells, which consequently perturbed the balance between neural progenitor cell expansion and differentiation in the forebrain of *Tcof1*$^{+/-}$ embryos. The depletion of neural progenitor cells specifically affected neurogenesis of upper layer neurons in association with reduced cerebral cortex size in *Tcof1*$^{+/-}$ pups (Sakai *et al.*, 2012).

Tcof1 regulates ribosome biogenesis for neural crest cell survival

Treacle co-localizes with upstream binding factor (UBF) and RNA polymerase I in the nucleolus where ribosomal RNA transcription takes place (Valdez *et al.*, 2004). Consistent with this intracellular localization, *TCOF1* knockdown showed that Treacle is important for rRNA transcription and pre-RNA processing, implying a role of TCOF1/Treacle in ribosome biogenesis (Valdez *et al.*, 2004, Gonzales *et al.*, 2005). Recently, mutations in *POLR1C* and *POLR1D* which encode subunits of both RNA polymerase I and III have also been identified in association with Treacher Collins syndrome (Dauwerse *et al.*, 2011). These findings imply a correlation between defects in ribosome biogenesis and the pathogenesis of TCS.

A role for *Tcof1* in ribosome biogenesis has also been elucidated in the *Tcof1*$^{+/-}$ mouse model of TCS (Dixon *et al.*, 2006, Jones *et al.*, 2008). *Tcof1*$^{+/-}$ embryos exhibit a reduction of rRNA, which presages a deficiency in mature ribosome production. The perturbation of ribosome biogenesis appears to correlate with a decrease in cell proliferation in the neuroepithelium and premigratory cranial neural crest cells in *Tcof1*$^{+/-}$ embryos. Consequently, it has been hypothesized that deficient ribosome biogenesis is insufficient to meet the cellular needs of highly proliferative cell populations such as neuroepithelial cells, and it is directly responsible for the high levels of apoptosis in the neuroepithelium and premigratory cranial neural crest cells in *Tcof1*$^{+/-}$ embryos (Dixon *et al.*, 2006, Jones *et al.*, 2008). The majority of craniofacial cartilage and bone is derived from cranial neural crest cells. Therefore, neuroepithelial apoptosis and consequent reduction in the number of cranial neural crest cells underpins the craniofacial anomalies in *Tcof1*$^{+/-}$ embryos.

Deficient ribosome biogenesis is known to trigger nucleolar stress activation of p53, which then induces the expression of pro-apoptotic genes (Rubbi & Milner, 2003). In *Tcof1*$^{+/-}$ embryos, p53 is activated and stabilized, which induces expression of pro-apoptotic genes, such as *Ccng1, Trp53inp1* and *Wig1* specifically in neuroepithelium and premigratory cranial neural crest cells (Jones *et al.*, 2008). These findings imply that haploinsufficiency of *Tcof1* results in a deficiency in ribosome biogenesis which triggers nucleolar stress induced activation and stabilization of p53. Activated p53 leads to apoptosis through the induction of pro-apoptotic gene expression. The correlation between ribosome biogenesis defects and p53-dependent apoptosis of neuroepithelial cells in *Tcof1*$^{+/-}$ embryos raised the possibility that suppressing apoptosis via inhibition of p53 might prevent craniofacial anomalies. Indeed, *in utero* treatment of *Tcof1*$^{+/-}$ embryos with pifithrin-α, a specific inhibitor for p53, reduced neuroepithelial apoptosis and ameliorated the craniofacial anomalies in a dose-dependent manner. Similarly, genetic removal of *p53* from the *Tcof1*$^{+/-}$ background (*Tcof1*$^{+/-}$; *p53*$^{+/-}$) also prevented the manifestation of craniofacial anomalies during embryogenesis. Furthermore, *Tcof1*$^{+/-}$; *p53*$^{+/-}$ pups were viable and also fertile. Surprisingly, the craniofacial skeleton in *Tcof1*$^{+/-}$; *p53*$^{+/-}$ embryos and newborn pups developed normally without restoration of ribosome biogenesis (Jones *et al.*, 2008).

Recently, the ubiquitination of Treacle was shown to be required for the regulation of translation, and subsequent cell-fate determination of ES cells (Werner *et al.*, 2015). The CUL3-KBTBD8 ubiquitin ligase complex monoubiquitinates Treacle, and this modification triggers a bridge between Treacle and NOLC1, another nucleolar protein involved in rRNA processing. The Treacle-NOLC1 complex connects RNA pol I with the H/ACA complex that catalyzes rRNA pseudouridylation and the SSU processome. This large complex appears to modify ribosomes and/or mRNA and induce distinct translational outputs, and as a result, ES cells differentiate towards a neural crest cell fate (Werner *et al.*, 2015). Thus, in addition to the production of ribosomes, Treacle may be also be important for their modification.

Novel function of *Tcof1*: suppressing apoptosis induced by oxidative stress

Rescue of the craniofacial skeleton in *Tcof1*$^{+/-}$; *p53*$^{+/-}$ embryos and newborn pups occurred without restoration of ribosome biogenesis (Jones *et al.*, 2008), suggesting that *Tcof1*/Treacle may also perform non-ribosome-associated functions. Consistent with this idea, three groups recently independently identified a role for Treacle in the DNA damage response/repair. In two studies, NBS1 as a bait, was shown to directly bind to Treacle in response to DNA damage induced by ionizing radiation in cultured cell lines (Ciccia *et al.*, 2014, Larsen *et al.*, 2014). In fact the translocation of NBS1 into nucleoli was dependent upon the phosphorylation of Treacle at threonine 210 by CK2 and at serine 1199 by ATM, a kinase which plays a central role in the DNA damage response (Fig. 1A). Treacle-dependent NBS1 translocation regulates silencing of RNA polymerase I-dependent rRNA transcription upon DNA damage (Fig. 2)(Ciccia *et al.*, 2014, Larsen *et al.*, 2014). It has also been reported that a small fraction of Treacle (isoform c) forms DNA damage foci dependent on Poly ADP ribose polymerase (PARP), which is involved in single-strand DNA damage response/repair (Fig. 2)(Larsen *et al.*, 2014).

In the third study, Multidimensional Protein Identification Technology (MudPIT) was used to identify novel functions for Treacle via the comprehensive detection of Treacle interacting proteins (Sakai *et al.*, 2016). This approach resulted in identification of the MRNM (MDC1-RAD50-NBS1-MRE11) complex, which is an important mediator of double-strand DNA damage response/repair (Fig. 2). This study provided direct evidence that *Tcof1* functioned in DNA damage response/repair in vivo, and elucidated a correlation between deficient DNA damage repair and the apoptotic elimination of cranial neural crest cells in *Tcof1*$^{+/-}$ embryos. In support of this finding, Treacle was shown to localize to sites of DNA damage and form DNA damage foci in response to X-ray irradiation (Fig. 1B; −IR and +IR). DNA damage foci localization of Treacle is diminished by knockdown of MDC1 in irradiated HeLa cells, validating the idea that Treacle functions as a component of MRNM complex. Moreover, *Tcof1*$^{+/-}$ embryo-derived mouse embryonic fibroblasts (MEFs) exhibit a delay in DNA damage repair together with multinucleation which is characteristic feature of DNA damage repair deficiency. Collectively this data confirms Treacle is a novel component of the MRNM complex and is essential for DNA damage response/repair (Fig. 2). Interestingly, the loss of *TCOF1* affects the localization of BRCA1 to DNA damage foci without disruption of the BRCA1-A scaffold complex (Wu *et al.*, 2012). Although direct binding between Treacle and BRCA1 has not been detected, ECT2 which is a BRCA1 C-terminus (BRCT) domain containing protein, interacts with BRCA1 as well as Treacle (Woods *et al.*, 2012). Thus ECT2 may be a potential mediator of Treacle-BRCA1 binding. Mutations in BRCA1 are known to increase the risk of breast cancer (Miki *et al.*, 1994), thus *TCOF1*/Treacle may play a role in the suppression of tumor formation and cancer formation through its interaction with BRCA1. It will be important in the future to determine how Treacle influences BRCA1 localization for a better understanding of the regulation of the DNA damage response/repair machinery during embryogenesis.

Under conditions whereby DNA damage is so severe as to not be repairable, damaged cells are eliminated by p53-dependent apoptosis in order to suppress tumor formation (Kaina, 2003). In *Tcof1*$^{+/-}$ embryos, phosphorylated-Chk2 positive and γH2AX-positive DNA damaged cells are detected specifically in the neuroepithelium, from which neural crest cells are derived. Phosphorylated-Chk2 can stabilize p53 and consequently induce apoptosis (Chehab *et al.*, 2000, Shieh *et al.*, 2000, Hirao *et al.*, 2000) and consistent with these findings, p53 accumulates in the neuroepithelium of *Tcof1*$^{+/-}$ embryos. Furthermore, DNA damaged neuroepithelial cells are also labeled with apoptotic marker cleaved-Caspase3 (Sakai *et al.*, 2016). Thus, *Tcof1*/Treacle links DNA damage response/repair to the apoptotic elimination of neuroepithelial cells and progenitor neural crest cells in *Tcof1*$^{+/-}$ embryos.

Interestingly, neuroepithelial cells including progenitor neural crest cells endogenously generate reactive oxygen species (ROS) at relatively high levels compared with other tissues (Fig. 3A; control)(Sakai *et al.*, 2016). ROS are strong oxidants, suggesting that oxidative DNA damage occurs frequently in those cells. Indeed, *in utero* treatment of *Tcof1*$^{+/-}$ embryos with the strong antioxidant, N-acetyl-cysteine (NAC), suppresses DNA damage, preventing p53 accumulation and apoptosis (Fig. 3A; +NAC). Surprisingly, prenatal antioxidant supplementation with NAC reduced the incidence and severity of craniofacial anomalies. 30% of treated *Tcof1*$^{+/-}$ embryos were fully rescued and were morphologically indistinguishable from wild-type littermates (Sakai *et al.*, 2016). The mechanisms

underlying the craniofacial abnormalities observed in *Tcof1*$^{+/-}$ embryos, and prenatal restoration of craniofacial abnormalities based on its pathogenic mechanism are summarized in Fig.3B and 4B (non-NE, NE and NE +NAC). Briefly, perturbation of DNA damage repair leads to the apoptotic elimination of damaged neuroepithelial and neural crest cells in *Tcof1*$^{+/-}$ embryos, while apoptosis and subsequent craniofacial defects are ameliorated by quenching ROS with antioxidants in *Tcof1*$^{+/-}$ embryos. Taken together, *Tcof1* is an essential gene for the protection of neuroepithelial and neural crest cells from endogenous and exogenous oxidative stress induced DNA damage during normal craniofacial development. Oxidative stress induced DNA damage appears to generally occur during embryonic development, thus other congenital craniofacial and brain disorders may be also be affected or caused by endogenous ROS. Therefore, these findings shed new light on potential therapeutic avenues for prevention of TCS as well as other congenital craniofacial and brain birth defects of similar origin.

Although craniofacial abnormalities can be ameliorated by antioxidant supplementation, *Tcof1* mutant pups still exhibit nasal anomalies and die 2–3 days after the birth due to respiratory failure (Sakai *et al.*, 2016). Further improvement in the degree of rescue may be attainable by varying the concentration of antioxidants and through combinatorial antioxidant supplementation. For future application in clinical medicine, antioxidants such as NAC, vitamin C, polyphenol and resveratrol which are popular, non-toxic, readily available and inexpensive dietary supplements, may provide avenues for exploration. Prenatal antioxidant supplementation may protect against craniofacial, brain and other congenital anomalies similar to prenatal folic acid supplementation in the prevention of anencephaly and spina bifida as recommended by the U.S. Public Health Service and CDC.

The neuroepithelium, which exists in an endogenously high oxidative state compare to non-neuroepithelium (Fig. 4B; non-NE and NE) is also more sensitive to increased ROS than its surrounding tissues. Exposing wild type embryos to ROS generator, 3-nitropropionic acid (3-NP) and the oxidant H_2O_2 induces massive apoptosis in the neuroepithelium and presumptive neural crest similar to the apoptotic pattern observed in *Tcof1*$^{+/-}$ embryos (Fig. 4A)(Sakai *et al.*, 2016). High levels of endogenous ROS lower the threshold for oxidative stress-induced apoptosis, therefore neuroepithelial and neural crest cells are highly sensitive to exogenous ROS (Fig. 4B; non-NE +ROS andNE +ROS). Environmental factors such as chemicals, UV irradiation, alcohol, smoking and high-calorie diet (lipids, glucose) are strongly linked to increased risk and severity of congenital birth defects (Gilbert, 2003, Ornoy *et al.*, 2015). Furthermore, such environmental factors are known to induce the generation of ROS (Wright *et al.*, 1999, Ikehata & Ono, 2011, Zhao & Reece, 2005). Taken together, these findings suggest that the inter- and intra-familial variability in the TCS phenotype, may be influenced by exposure of embryos to environmental factor(s) during pregnancy. These studies of the pathogenesis of TCS illustrate the involvement of environmental factors in the variability of craniofacial abnormalities.

Perspectives

High levels of ROS in the neuroepithelium is a fundamental contributor to the pathogenesis of TCS. This raises the questions of what is the source of endogenous ROS, and why do

neuroepithelial cells generate higher levels of ROS than their surrounding tissues. Experimental animals are kept in highly regulated and well controlled laboratory animal facilities, and are not exposed to radiation or high-calorie diets. However, there are many alternative mechanisms for the generation of ROS. One such source of ROS is the electron transport chain, which generates ATP during respiratory metabolism within mitochondria. Electrons derived from the oxidation of metabolic intermediates lead to the generation of ROS (Kirkinezos & Moraes, 2001).

Interestingly, the cranial region exhibits a relatively high uptake of glucose compared to trunk region (Hunter & Tugman, 1995). This further illustrates the contribution of respiratory metabolism to endogenous ROS generation. In addition, our recent findings suggest that some genes involved in respiratory metabolism exhibit neuroepithelium-specific expression in early stages of mouse embryo development (D. Sakai, unpublished data). This further supports the correlation between respiratory metabolism and endogenous ROS generation. Elucidating the precise sources of endogenous and exogenous ROS will be very important in the development of future therapeutic approaches for preventing or treating TCS.

Although, antioxidant treatment can ameliorate craniofacial abnormalities in *Tcof1*$^{+/-}$ embryos, high doses of antioxidants can lead to embryonic lethality (D. Sakai, unpublished data). This implies that specific levels of ROS might be required for normal craniofacial and brain development. In agreement with this idea, it has become evident that ROS can function as a signaling molecule to regulate many biological processes during embryonic development (Covarrubias *et al.*, 2008, Le Belle *et al.*, 2011). For example, the subventricular zone of the cortex in E14.5 mouse embryos exhibits elevated ROS and reducing endogenous ROS affects the proliferation of neural progenitor cells *in vivo*. Furthermore, the PI3 kinase-Akt-mTOR pathway is activated by endogenous ROS which also regulates neural stem and progenitor cell proliferation. Thus it will be very interesting to determine in the future whether endogenous ROS activates similar pathways and enhances proliferation in the neuroepithelium during early embryogenesis as a further step towards a better understanding of normal and abnormal craniofacial development.

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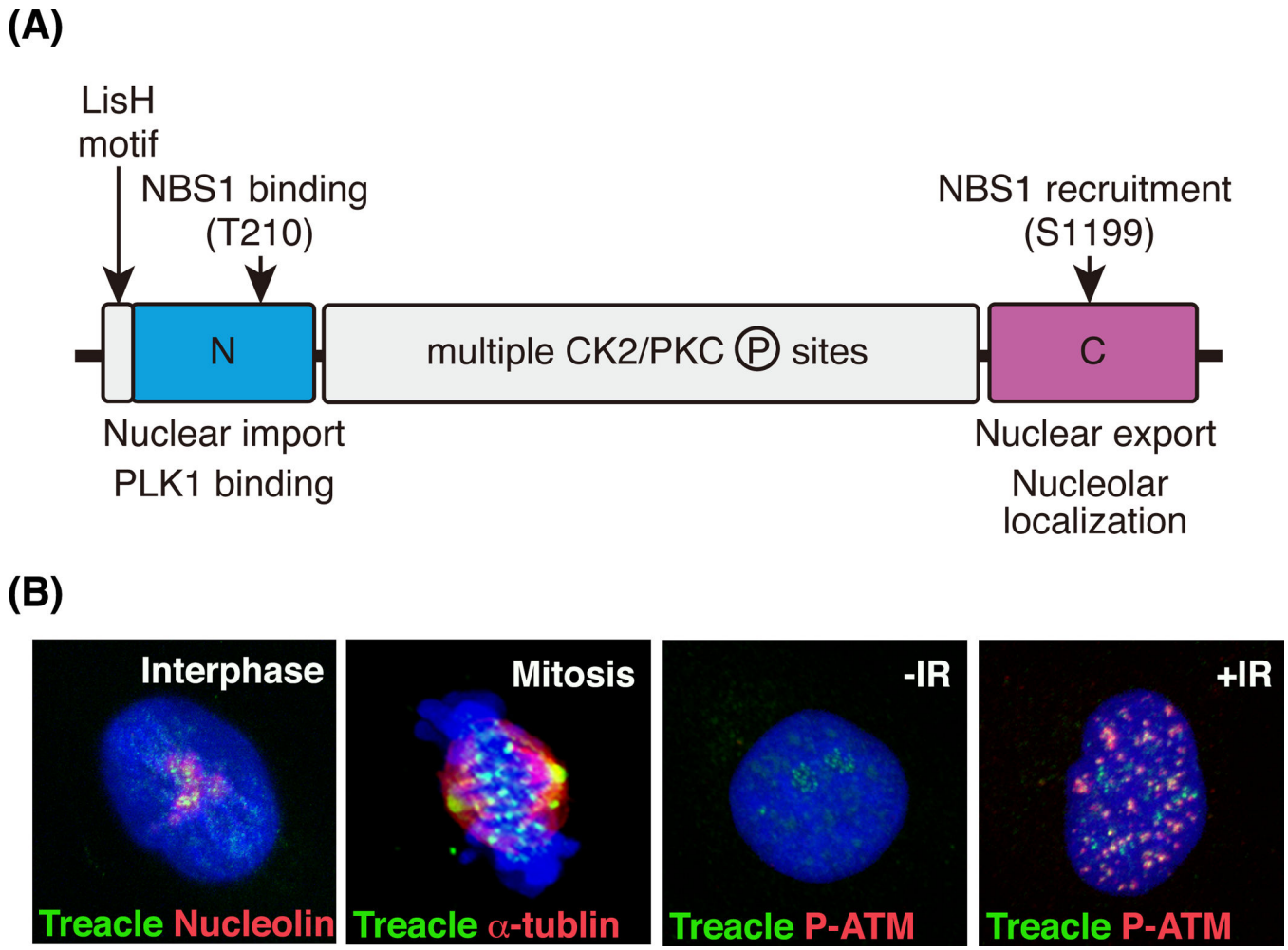

Fig.1.

Protein domain structure and dynamic cellular localization of Treacle. (A) Domain structure of Treacle is shown. Treacle consists of three regions, N-terminal, central and C-terminal region. A LisH motif is located at the N-terminus of Treacle. Threonine 210 is phosphorylated by CK2 and required for NBS1 binding. Serine 1199 is phosphorylated by ATM kinase and required for NBS1 recruitment into nucleolus. (B) Treacle localizes in the nucleolus during the interphase of the cell cycle (Interphase). Nucleoli are visualized by nucleolin immunostaining. During the mitotic phase, Treacle localizes to the centrosome and kinetochore (Mitosis). The mitotic spindle is marked by α-tubulin immunostaining. Nucleolar Treacle in non-irradiated cell (−IR) disperses in nucleus and forms DNA damage foci co-labeled with DNA damage response protein, phosphorylated-ATM (P-ATM, +IR).

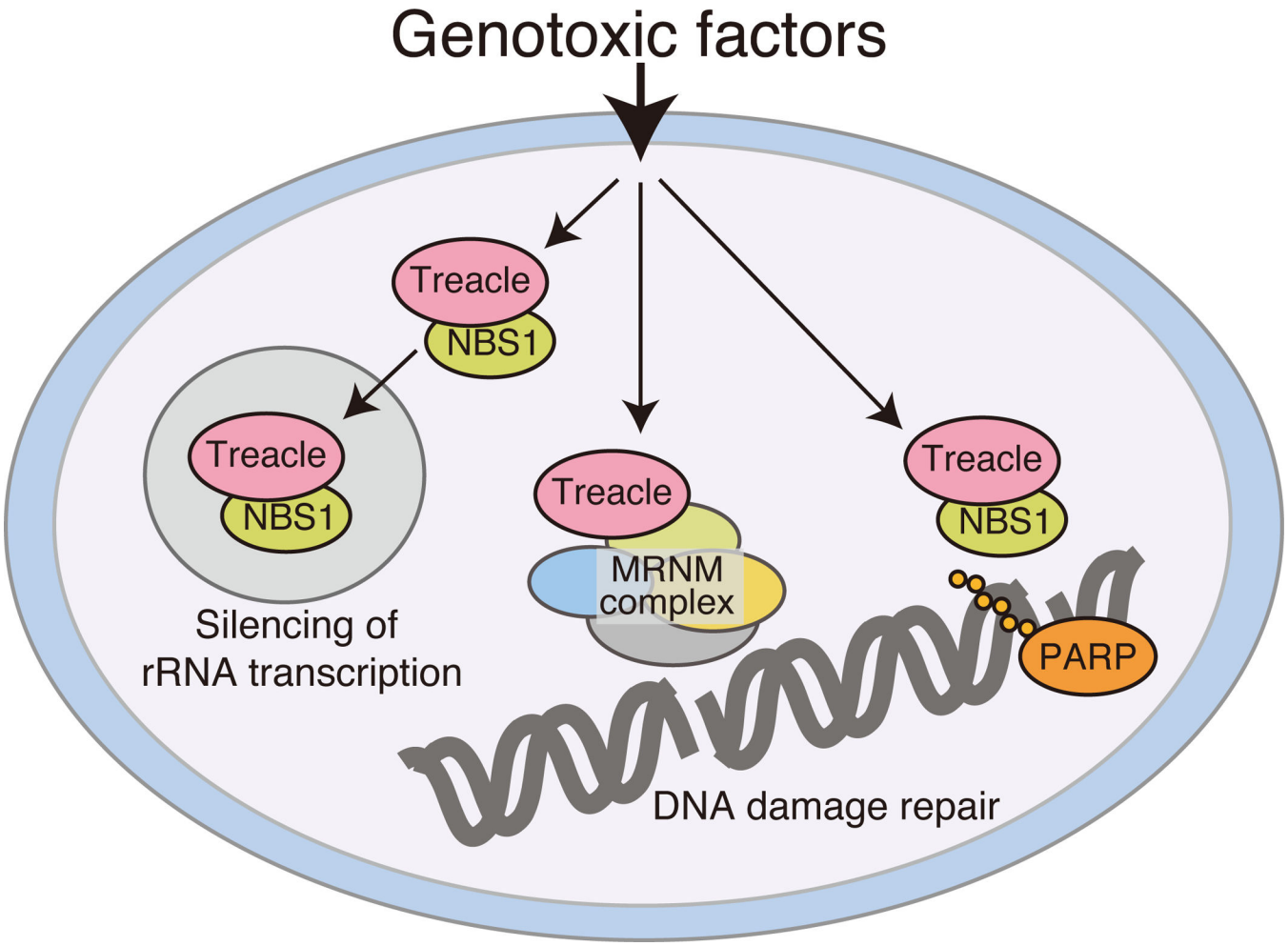

Fig.2.

Multiple functions of Treacle in response to DNA damage. DNA damage response protein NBS1 is recruited into the nucleolus in response to DNA damage. Nucleolar recruitment is dependent on Treacle, and an NBS1-Treacle complex silences rRNA transcription through the inhibition of RNA polymerase I function. Treacle also associates with DNA damage response and subsequent repair with an important DNA damage response complex MDC1-RAD50-NBS1-MRE11 complex. Treacle isoform C regulates PARP-dependent single-strand DNA damage repair.

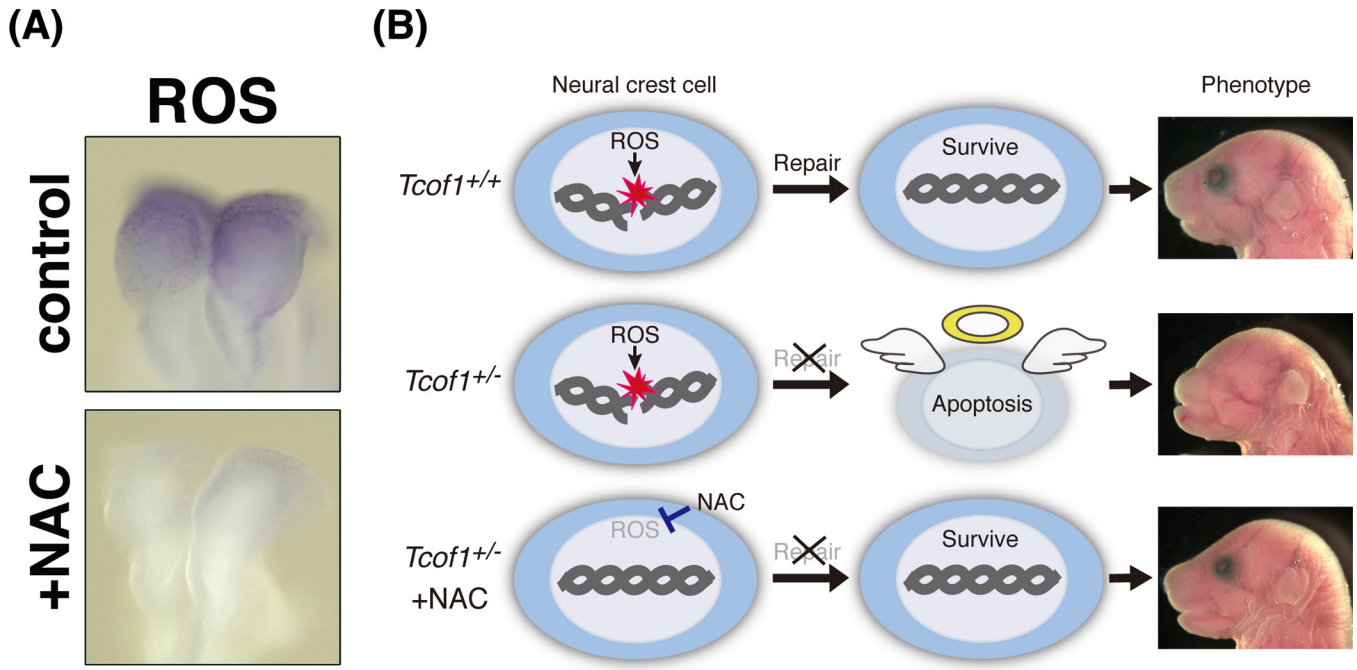

Fig. 3.

Antioxidants reduce endogenous ROS and rescue craniofacial abnormalities. (A) Wild type embryos were treated with a strong antioxidant, NAC, via intraperitoneal injection of pregnant females at E8.5. After a 1hour incubation, embryos were subjected to NBT staining to visualize ROS. NAC effectively scavenged ROS. (B) Summary of the mechanism of preventing craniofacial abnormalities in *Tcof1*$^{+/-}$ embryos. Endogenous ROS frequently damages chromosomal DNA. Thus, the DNA damage response/repair machinery is dependent on Treacle which is required for prevention of oxidative DNA damage and subsequent apoptosis. In *Tcof1*$^{+/-}$ embryos, progenitor neural crest cells undergo apoptosis due to their perturbed ability to repair DNA. In contrast, scavenging of ROS via antioxidants such as NAC prevents DNA damage, reduces apoptosis and rescues the craniofacial abnormalities in *Tcof1*$^{+/-}$ embryos.

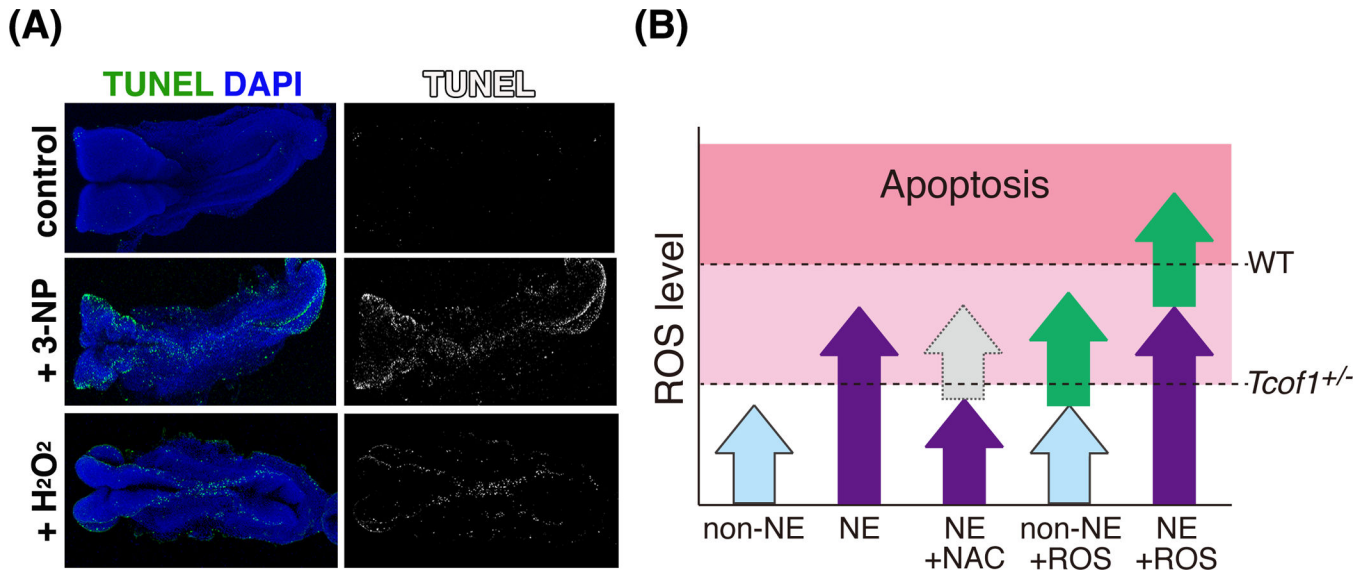

Fig. 4.

Induction of neuroepithelial apoptosis with excess ROS in wild type embryos. (A) Wild type E8.5 whole embryos were cultured in the presence of a strong oxidant H_2O_2 and ROS-generator, 3-NP. Apoptosis was detected by whole-mount TUNEL staining. Apoptosis was induced in neuroepithelium, especially at the dorsolateral edges where neural crest is formed. (B) Schematic representation of the relationship between ROS levels and induction of neuroepithelial apoptosis. Arrows indicate levels of ROS in non-neuroepithelium (light blue), neuroepithelium (purple), quenching by NAC (gray), addition or induction by 3-NP (green). Dotted lines indicate the threshold for oxidative stress-induced apoptosis in wild type (WT) and *Tcof1* heterozygous mutant (*Tcof1*$^{+/-}$) embryos, respectively.