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REVIEW

Redox regulation of Wnt signalling via nucleoredoxin

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Abstract
Numerous studies indicate that reactive oxygen species (ROS) are not merely cellular by-products of respiration, but are able to modulate various signalling pathways and play certain physiological roles. Recent studies have revealed the importance of translating ROS-generation to activation/suppression of specific signalling pathways. The Wnt signalling pathway, which is essential for early development and stem cell maintenance, is also regulated by ROS. A thioredoxin-related protein, nucleoredoxin (NRX), governs ROS-stimulated Wnt signalling in a temporal manner. NRX usually interacts with Dishevelled (Dvl), an essential adaptor protein for Wnt signalling, and blocks the activation of the Wnt pathway. Oxidative stress causes dissociation of NRX from Dvl, which enables Dvl to activate the downstream Wnt signalling pathway. This study also presents the latest research findings on NRX and its related molecules

Keywords: *Wnt, nucleoreodoxin (NRX), thioredoxin (TRX)*

Overview of the Wnt signalling pathways

The Wnt signalling pathway is highly conserved throughout evolution. It is essential for early development. In many organisms, it has been shown that abrogation of the Wnt signalling pathway results in embryonic malformation.

There are two major pathways elicited by Wnt ligands, the Wnt/β-catenin signalling pathway and the Wnt/planar cell polarity (PCP) pathway. Research into the Wnt/β-catenin signalling pathway was first initiated by Sharma [1]. He isolated a *Drosophila* mutant called '*wingless*', i.e. a fly with no wings. Further genetical and biochemical analyses in *drosophila* and in mammalian cell cultures established the existence of a signalling pathway triggered by Wnt ligands. Meanwhile, Nusse et al. [2] identified a new gene '*Int-1*' that was ectopically activated by the proviral insertion of the mouse mammary tumour virus (MMTV), which causes mammary tumours in mice. Three years later, Rijsewijk et al. [3] discovered *Int-1* to be a mammalian homologue of *Drosophila wingless*. The name 'Wnt' is, thus, derived from *wingless* and *Int-1*.

As described above, the Wnt/β-catenin signalling pathway is deeply involved in cancers. Adenomatous polyposis coli (APC), an important suppressor of the Wnt/β-catenin signalling pathway, is encoded by the gene responsible for familial adenomatous polyposis (FAP). FAP results in the formation of numerous polyps, some of which develop into colorectal tumours [4]. Mutations in several components of the Wnt/β-catenin signalling pathway (*β-catenin*, *Axin1/2* and *TCF4*) have been also found in cancers [5]. In addition, gene amplification of *Dvl* [6] and epigenetic silencing of sFRP, a suppressor of Wnt/β-catenin signalling outside the cell, are found in various tumours [7].

The Wnt/β-catenin signalling pathway is also known to be important for stem cell maintenance [8]. It was reported that both Wnt3a-conditioned media and BIO, an inhibitor for glycogen synthase kinase 3β(GSK3 β), which activates Wnt/β-catenin signalling, are sufficient for maintaining self-renewal

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and pluripotent capabilities of embryonic stem (ES) cells [9]. Besides ES cells, it has been reported that Wnt/β-catenin signalling is important for maintenance of many other stem cell types including hematopoietic and mesenchymal stem cells [10,11].

Current understanding of the Wnt/β-catenin signalling is summarized in Figure 1. When the Wnt ligand is absent, β-catenin is efficiently phosphorylated by a serine/threonine kinase, GSK3 β, a process facilitated by Axin and APC (therefore, APC/Axin/GSK3β is sometimes called 'β-catenin destruction complex'). Phosphorylated β-catenin is targeted for ubiquitination via β-TrCP and is rapidly degraded. In this way, cytosolic β-catenin is maintained at a low level. When Wnt ligand binds to its receptor, Frizzled (Fzd), the phosphorylation of β-catenin by APC/Axin/GSK3 β complex is suppressed. Dvl serves as an adaptor protein that links Fzd to APC/Axin/GSK3 β complex. Upon Wnt stimulation, Dvl is recruited to the plasma membrane via Fzd and co-receptor, LRP5/6. Several recent reports showed that Dvl polymerizes and recruits Axin, leading to the suppression of the APC/Axin/GSK3 β complex [12,13]. This allows β-catenin to escape from degradation and accumulate in the cytosol, from where it translocates into the nucleus. Nuclear β-catenin binds to a transcription factor, TCF/LEF, and controls numerous target genes, including *c-Myc*, *CyclinD1* and *Axin2* [14–16].

The other major Wnt-dependent pathway, the Wnt/planar cell polarity (PCP) pathway, governs multiple phenomena, such as orientation of hairs and bristles in *Drosophila*, neuronal polarity in mammalian neurons and gastrulation movements in vertebrates [17–20]. The Wnt/PCP pathway was also first discovered in *Drosophila* studies. Vinson and Adler [21] found a mutant fly with misoriented cuticle hairs and bristles, which they named '*frizzled*'. The mutant protein (which is called by the same name) was later identified as a Wnt receptor and a component of both the Wnt/β-catenin pathway and the Wnt/PCP pathway [22]. Like the Wnt/β-catenin pathway, the existence of the Wnt/PCP pathway was also established through identification of various genes that are genetically associated with *frizzled* in *Drosophila*. Notably, Wnt, Fzd and Dvl are common components in both the Wnt/β-catenin pathway and the Wnt/PCP pathway, while other factors such as Van Gogh/Strabismus (Vang/Stbm), Rho and c-Jun are only involved in the Wnt/PCP pathway [23–27]. Therefore, Dvl has been described as a branch point of these two pathways [26,28,29].

Dishevelled was also first identified in *Drosophila* in 1987 [30]. The mammalian counterpart is called *Dvl* and there are three *Dvl* isoforms (*Dvl1-3*) [31–33]. All Dvl proteins have three conserved domains: the NH_2-terminal DIX (Dvl/Axin) domain [34], the central basic-PDZ (PDZ stands for Post-synaptic density-95 (PSD-95), Discs large (Dlg), Zona occludens (ZO1)) domain [35] and the COOH-terminal DEP (Dvl, EGL-10, pleckstrin) domain [36]. These three domains bind to different proteins and are required for Dvl function. It is shown that the DIX and the basic-PDZ domains are important for the activation of the Wnt/β-catenin pathway and the basic-PDZ and the DEP domains are necessary for the Wnt/PCP

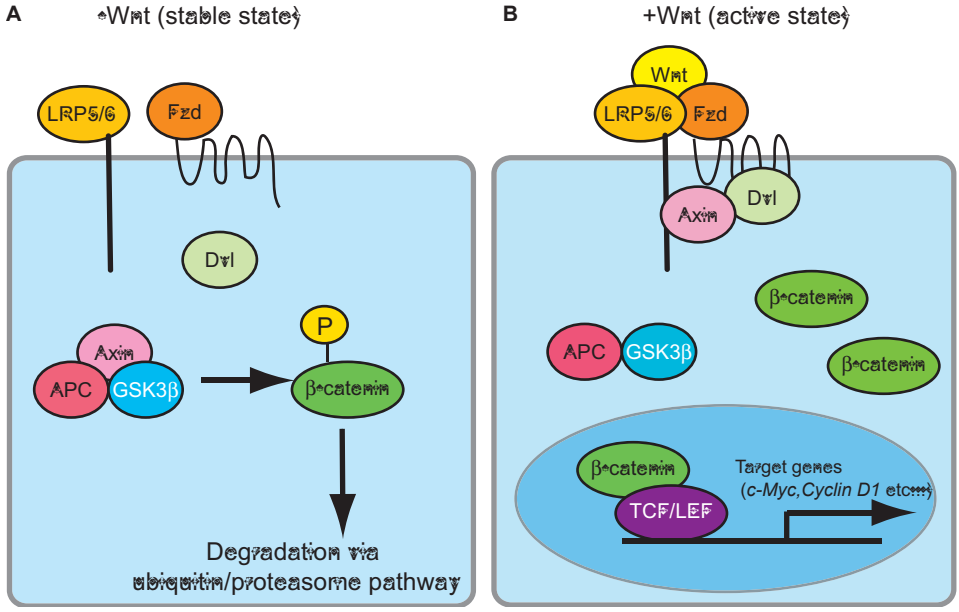

Figure 1. Model of Wnt/β-catenin signal transduction. (A) At the resting state (without Wnt ligand), β-catenin is efficiently phosphorylated by the β-catenin destruction complex, composed of APC, Axin and GSK3 β, and rapidly degraded via ubiquitin/proteasome pathway. (B) Wnt ligand binds to the receptor Frizzled (Fzd) and LRP5/6, which recruits Dvl and Axin to prevent phosphorylation of β-catenin by the β-catenin destruction complex. β-catenin escapes degradation and accumulates in the cytosol, resulting in the translocation to the nucleus. In the nucleus, it binds to the transcription factor, TCF/LEF, to activate the expression of various target genes.

pathway, which ensures that Dvl exists at the crossroad of these two pathways.

Knockout mice have been generated for each of the three Dvl isoforms. The first characterization of the *Dvl1*-knockout mouse was reported in 1997; this study reported that these mice develop normally but show abnormalities in social behaviour [37]. A direct link between these abnormalities and the loss of Dvl1 has not been demonstrated; however, it is reported that hippocampal neurons obtained from *Dvl1*-knockout mice show defects in dendrite development [38]. Approximately half of the *Dvl2*-knockout mice died perinatally and these mice showed cardiovascular abnormalities [39]. *Dvl3*-knockout mice also died perinatally and had defects similar to those of *Dvl2*-knockout mice. Expression of a *Dvl3* transgene was found to partially rescue this phenotype [40]. The phenotypic differences between the three *Dvl* knockout mice may be explained by differences in the expression patterns of these three molecules. It is reported that Dvl1 expression is relatively strong in the central nervous system [31], while Dvl2 and Dvl3 are expressed more ubiquitously [33,41]. In fact, Etheridge et al. [40] demonstrated that *Dvl3*-knockout mice can also be rescued by expression of a *Dvl1* or *Dvl2* transgene. Furthermore, *Dvl1, 2* or *Dvl2, 3* double knockout mice show more severe phenotypes such as neural tube closure defects, suggesting the functional redundancy of these proteins. Taken together, these studies indicate that all three Dvl isoforms play fundamental roles during mouse development, which highlights the importance of elucidating their mechanism of action.

Identification of nucleoredoxin (NRX) as a novel interacting partner of Dvl

In order to determine the molecular mechanisms underlying Dvl function, we planned to perform a comprehensive proteomic search for novel Dvl-interacting proteins. For this purpose, we first generated an NIH3T3 murine fibroblast-derived cell line stably expressing FLAG-tagged Dvl1 (FLAG-Dvl1) or GFP as a control. We collected cell lysates and performed immunoprecipitation with anti-FLAG antibodies. By using silver staining, we observed several bands that were specific to immunoprecipitates of lysate obtained from FLAG-Dvl1-expressing cells. The most abundant protein was subjected to mass spectrometry and identified as NRX [42].

NRX was first identified by Kurooka et al. [43], as a gene adjacent to the *nude* (*Foxn1*, *Whn* or *Hfh11*) gene locus. They cloned this gene and found that the protein product localized to the nucleus when it is over-expressed in cultured cells. Therefore, this molecule was named 'nucleo'-redoxin, being a nuclear member of the thioredoxin (TRX) family. In contrast, our results using anti-NRX antibody revealed that endogenous NRX mainly exists in the cytosol, suggesting that NRX may shuttle between the cytosol and the nucleus. Interestingly, Dvl is also reported to exist in both the cytosol and the nucleus [44]. More detailed analyses of NRX localization are required.

As mentioned, NRX is a member of the thioredoxin (TRX) family of proteins. TRX was first identified as an electron donor for *E. coli* ribonucleotide reductase [45]. TRX is highly conserved throughout species in both prokaryotes and eukaryotes and is well known as a major antioxidant enzyme. TRX and its family of proteins possess a typical catalytic motif, Cys-X-X-Cys, and TRX exerts a disulphide bond-reducing activity. Numerous target proteins of the TRX enzymatic activity have been reported, such as methionine sulphoxide reductase, NF-κB, Ref-1, ribonucleotide reductase and 2-Cys peroxiredoxins (PRXs) [46–51]. Among them, 2-Cys PRXs have been extensively studied and characterized. 2-Cys PRXs directly reduce hydrogen peroxide (H_2O_2), peroxynitrite and organic hydroperoxides and protect cells from oxidative stress. During this reaction, 2-Cys PRXs become oxidized and TRX reduces and reactivates 2-Cys PRXs. TRX is then reduced by thioredoxin reductase (TrxR), which mediates an electron

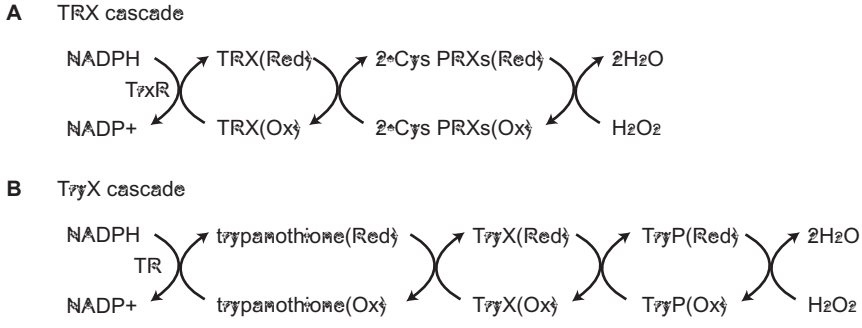

Figure 2. TRX and TryX cascades. (A) 2-Cys PRXs are H_2O_2-scavenging enzymes. Oxidized 2-Cys PRXs are reactivated by TRX with electrons derived from NADPH through TrxR. (B) In trypanosomes, tryparedoxin peroxidase (TryP) eliminates H_2O_2. Oxidized TryP is reduced by TryX, with electrons donated by trypanothione. NADPH reduces trypanothione through catalytic activity of trypanothione reductase (TR).

transfer from NADPH to oxidized TRX. This string of reactions is called the TRX cascade and is known as one of the schemes to scavenge H_2O_2 from cells (Figure 2). Besides these specific targets, TRX also reduces irregular protein disulphide bonds caused by oxidative stress.

To date, there are more than 20 TRX family members. Among them, NRX, Rod-derived cone viability factor (RdCVF) and Chromosome 9 open reading frame 121 (C9orf121, also called RdCVF2) can be classified in the same sub-family, based on the relatively high similarity in their amino acid sequence [52,53]. The TRX domains of these three proteins are slightly different from the conventional TRX domain and more closely resemble that of tryparedoxin (TryX), a TRX-like protein identified in the parasite, trypanosomatid [54] (Figure 3). Trypanosomatids are protozoan parasites which cause various serious diseases, such as visceral Leishmaniasis, sleeping sickness and the Chagas' disease and kill thousands of people a year [55]. While other organisms use mainly TRX and glutathione systems to maintain redox homeostasis, trypanosomatids do not have such systems. Instead, they have a unique redox homeostasis system utilizing trypanothione, a spermidine-glutathione conjugate only found in trypanosomatids (Figure 2). NRX, RdCVF

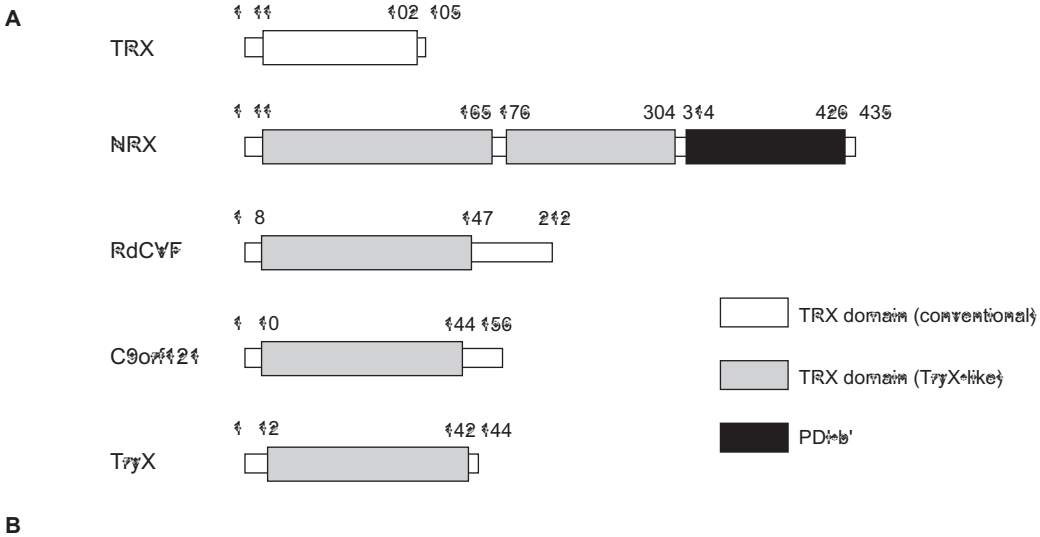

Figure 3. Similarities between TRX, NRX, RdCVF, C9orf121 and TryX proteins. (A) Schematics of the human TRX (NP_003320), NRX (NP_071908), RdCVF (NP_612463), C9orf121 (NP_001155097) and TryX of *Trypanosoma brucei* (XP_843968). The amino acid numbers of each protein and domain are also shown. (B) The sequences of the conserved regions in the proteins shown in (A) (conventional TRX domain for TRX and TryX-like domains for the others) are aligned using clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Gap regions that specifically exist in TRX are boxed. The conserved catalytic sequences of Cys-X-X-Cys are in grey. The asterisk (*), colon (:), and period (.) denote identical, conserved substitutions and semi-conserved substitutions, respectively.

and C9orf121 possess, in common, an uncharacterized domain that is similar to TryX, which is called TryX-like domain. RdCVF and C9orf121 contain one TryX-like domain, while NRX possesses two TryX-like domains. Of the two TryX-like domains in NRX, the consensus TRX motif of the NH_2-terminal TryX-like domain is altered to Ser-Ala-Pro-Cys. NRX also bears a PDI-b'-like domain in its COOH-terminus. The PDI-b' domain is required for substrate recognition during the catalytic reaction of protein disulphide isomerase (PDI) proteins, another sub-group of the TRX family, which functions in the ER to facilitate the proper folding of proteins [56]. As NRX possesses oxidoreductase activity, it is possible that NRX also utilizes its PDI-b'-like domain as a substrate recognition domain in cells.

RdCVF (also known as TRX-like 6) was identified as a protein which enhances the viability of cone cells [57]. RdCVF is implicated in upregulation of NF-κB activity [58] and protects tau from oxidative stress [59]. Such molecular functions may be important for its cellular function as a cone viability factor. C9orf121 also contains a TryX-like domain and is also reported to enhance cone viability like RdCVF [53].

NRX suppresses the Wnt/β-catenin signalling pathway in a redox-dependent manner

To elucidate the action of NRX against the Wnt/β-catenin signalling pathway, we performed various over-expression experiments in cultured cells. We found that (i) β-catenin accumulation caused by Dvl over-expression is suppressed by NRX co-expression, but not by TRX or a mutant form of NRX (in which both cysteine residues in the TRX motif are mutated to serine, preventing it from binding to Dvl), (ii) β-catenin accumulation caused by Wnt3a ligand treatment is prevented in wild-type NRX-over-expressing cells, but not in the cysteine mutant NRX-over-expressing cells, (iii) TCF/LEF reporter activity upregulated by Dvl over-expression is suppressed by NRX co-expression, which cannot, however, inhibit upregulation by over-expression of constitutively active mutant of β-catenin [42,52]. Taken together, NRX is an inhibitor of Wnt/β-catenin signalling at the level of Dvl and its inhibitory effect is exerted via binding to Dvl. We also performed loss-of-function analyses of NRX utilizing RNA interference (RNAi). By reporter assays and RT-PCR, we confirmed that NRX is a negative regulator of the Wnt/β-catenin pathway.

We also discovered that ectopic expression of NRX leads to the dephosphorylation of Dvl. Dvl-phosphorylation reportedly correlates with the Wnt/β-catenin pathway activity [60], supporting our above-mentioned results showing that NRX functions as a negative regulator of the Wnt/β-catenin pathway. The mechanism of how NRX down-regulates the phosphorylation status of Dvl has not been identified. It should be noted here that NRX also binds to $PP2A_C$, a catalytic sub-unit of protein phosphatase 2A (PP2A), and competes with PR55, a regulatory sub-unit of PP2A [61]. It may be possible that Dvl dephosphorylation is mediated by PP2A, which is recruited to Dvl via NRX. In fact, it has been reported that PP2A plays an inhibitory role in Wnt/β-catenin signalling [62,63]. It is also possible that NRX regulates PP2A phosphatase activity via its oxidoreductase activity. There is a report that $PP2A_C$ is inactivated by oxidation and is reactivated by reducing agents [64].

As NRX is a TRX family protein and the mutant protein of its catalytic cysteine does not bind to Dvl, it was hypothesized that the association between Dvl and NRX could be redox-dependent. Therefore, *in vitro* pull-down experiments were performed with GST-tagged Dvl1 and His-tagged NRX proteins under reduced and oxidized conditions with dithiothreitol (DTT) and H_2O_2, respectively. The interaction between these two proteins was strengthened in the reduced conditions and weakened by H_2O_2 in a dose-dependent manner [42]. We also treated cells with H_2O_2 and performed immunoprecipitation experiments on their lysates. As we expected, the complex formation between Dvl and NRX is weakened by H_2O_2 treatment. Therefore, we concluded that Dvl and NRX interact in a redox-dependent manner. Treatment of Dvl and NRX with DTT or H_2O_2 by itself showed that NRX is responsible for this redox-dependent interaction. It is known that there is a small but significant change in the protein structure when TRX is subjected to oxidizing or reducing conditions [65]. Future structural analyses of NRX may reveal whether NRX also shows a similar redox-dependent conformational alteration and how it contributes to the redox-dependent interaction between NRX and Dvl.

The two findings that (a) Dvl and NRX interact in a redox-dependent manner and (b) NRX suppresses the Wnt/β-catenin pathway, prompted us to set a hypothesis that the Wnt/β-catenin pathway may be redox-regulated through NRX. When we treated cells with H_2O_2, a significant upregulation of the Wnt/β-catenin pathway, as evidenced by β-catenin accumulation and target gene expression, was observed. This redox-dependent upregulation of the Wnt/β-catenin pathway did not occur in NRX-RNAi cells, showing that this event is mediated through NRX.

Apoptosis signal-regulating kinase 1 (Ask1) also binds to TRX in a redox-dependent manner and regulates downstream signalling, leading to apoptosis [66,67]. Under normal (reducing) cellular conditions, TRX binds to Ask1 and prevents its activation. Oxidative stress converts TRX to its oxidized form which dissociates from Ask1. TRX-free Ask1 becomes

activated and stimulates the downstream 'stress signal cascade' via its kinase activity. As NRX is also a TRX family protein, the redox-dependent activation of Wnt/β-catenin pathway could be explained by an analogous mechanism (Figure 4).

What could be the physiological function of redox-dependent activation of the Wnt/β-catenin pathway? Several groups have reported that Wnt/β-catenin signalling is suppressed by chronic oxidative stress (more than 12 h of H_2O_2 treatment) [68–70]. In contrast, the above-mentioned H_2O_2 treatment-dependent activation of Wnt/β-catenin signalling was rather a rapid and transient event (5–180 min). Therefore, the redox-dependent activation of Wnt/β-catenin signalling under physiological conditions should also be temporally or spatially limited. One possible scenario is that acute stimulation of the Wnt/β-catenin pathway in response to a mild and short-term oxidative stress prevents the unnecessary activation of apoptotic signalling pathways. In general, when mild stress is applied to cells, they tend not to choose apoptosis but rather resist/adapt to it. For example, in the case of DNA damage, cells choose cell cycle arrest followed by DNA repair or activation of the apoptotic signalling pathway in a manner dependent on the extent of the DNA damage [71]. In fact, activation of Wnt/β-catenin signalling induces the expression of various anti-apoptotic genes and protects cells from apoptosis [72,73].

Another possibility is that the Wnt/β-catenin pathway itself generates ROS as a means of signal propagation. Growth factors are known to induce the generation of ROS in cells as intracellular mediators for downstream signalling [74]. Furthermore, recent reports claim that Rac, a firmly established activator of NADPH oxidase that generates superoxide in phagocytes, is important not only for the Wnt/PCP pathway but also for the Wnt/β-catenin pathway [75]. Therefore, Wnt stimulation may also actively generate and utilize ROS as either essential or auxiliary factors for signal transduction.

As noted above, chronic oxidative stress results in down-regulation of the Wnt/β-catenin pathway. This is thought to be due to the redox-dependent interaction between β-catenin and FOXO, a transcription factor, the activation of which induces the transcription of various anti-oxidant genes [68,69,76]. When FOXO binds to β-catenin, it competitively inhibits TCF/LEF binding to β-catenin, which may explain the observed down-regulation of Wnt/β-catenin signalling under conditions of chronic oxidative stress. In this process, it has been shown that β-catenin is an essential cofactor for the function of FOXO as a transcription factor [76]. Thus, accumulation of β-catenin following dissociation from NRX in response to H_2O_2 treatment might function to upregulate FOXO transcriptional activity when cells are challenged by chronic oxidative stress (Figure 4).

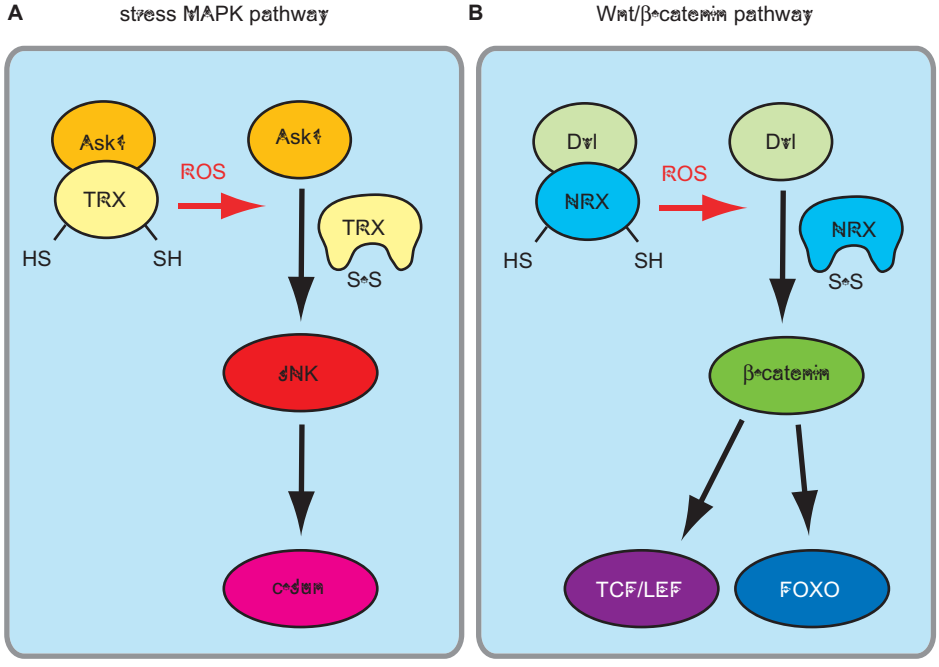

Figure 4. Redox-dependent modulation of stress kinase pathway and Wnt/β-catenin pathway. (A) Stimulation of cells with ROS induces oxidation of TRX either directly or through 2-Cys PRXs and formation of intramolecular disulphide bond. Oxidized form of TRX dissociates from Ask1, which results in the activation of Ask1 and the downstream stress mitogen-activated protein kinase (MAPK) pathway. (B) ROS also induces dissociation of NRX from Dvl, which results in the accumulation of β-catenin. β-catenin may associate either with TCF/LEF (transient stress) or FOXO (chronic stress) to activate the expression of various target genes.

NRX is an essential component in early development

To understand the role of NRX at the level of the organism, we performed experiments with the African clawed frog, *Xenopus laevis*. It has been shown that ectopic activation of Wnt/β-catenin signalling in the ventral marginal zone (VMZ) of embryos results in duplicate axis formation [77]. When we performed co-injection experiments with *Dvl* and *NRX* mRNAs, we found that duplicate axis formation induced by *Dvl* mRNA injection was clearly suppressed by *NRX* mRNA injection [42]. In *Xenopus*, Wnt/β-catenin signalling is also essential for anterior-posterior (AP) axis formation [78,79]. Downregulation of the *Xenopus* NRX homologue (MGC84045) by injecting morpholino antisense oligonucleotide (MO) resulted in aberrant head formation, which was rescued by coinjecting mRNA of NRX or other negative regulators of Wnt/β-catenin signalling (GSK3 β or a dominant negative form of TCF). We also confirmed that embryos injected with NRX MO showed an increase in the Wnt/β-catenin signalling activity and the expression of anterior marker *Bf-1* was considerably reduced. Collectively, these data clearly indicate that NRX functions as a negative regulator of Wnt/β-catenin signalling, not only in culture cells but also in *Xenopus* embryos.

To investigate the role of NRX in the Wnt/PCP pathway, another major Wnt-induced pathway, we injected *NRX* mRNA or MO into the dorsoanimal region of fertilized *Xenopus* eggs. We found both these embryos to display a bent-axis phenotype, typically observed in embryos with abnormal Wnt/PCP pathway activity [80]. Thus, NRX appears also to be important for the Wnt/PCP pathway. When we co-injected *Dvl* mRNA and *NRX* mRNA, we found that the bent-axis phenotype caused by *Dvl* mRNA was partially cancelled by *NRX* mRNA, suggesting that NRX affects the Wnt/PCP pathway by inhibiting Dvl function. The bent-axis phenotype of *Xenopus* embryos induced by perturbation of the Wnt/PCP pathway is a result of a convergent extension defect. By using Alexa© fluorescent dyes and animal cap assays to directly observe extension movement, we confirmed that embryos injected with *NRX* mRNA or *NRX* MO have defects in convergent extension. Activation of the Wnt/PCP pathway in mammalian cultured cells results in elevated phosphorylation of c-Jun. Indeed, c-Jun phosphorylation elevated by Dvl-expression is suppressed by NRX co-expression. In contrast, NRX cannot suppress c-Jun phosphorylation stimulated by the dominant active form of Rac1, Rac1G12V, which functions downstream of Dvl. These data suggest an evolutionarily conserved role of NRX in the regulation of the Wnt/PCP pathway through Dvl.

It was recently reported that in zebrafish H_2O_2 is generated at the wound and functions as a signal for the recruitment of leukocytes *in vivo* [81]. In general, such migratory behaviour is confined to specific types of cells, such as leukocytes, in adult animals, but a variety of cells dramatically migrate in developmental stages (e.g. neural crest cells) to cause morphogenesis. Therefore, ROS might also contribute to the migration of these cells in a fashion similar to the case of the zebrafish leukocytes. In such situations, NRX would be oxidized, resulting in the activation of Rac/Rho via the Wnt/PCP pathway, which drives cell migration. Indeed, it is reported that migration of culture cells during wound healing is upregulated by Wnt stimulation and Dvl and Rho, but not β-catenin, play important roles in this process [82].

Mice carrying mutations in the *NRX* gene were very recently generated and described. The group led by Justice generated various mutant mice by ENU-based random mutagenesis and found one mouse strain to be a hypomorphic mutant of *NRX* [83]. These *NRX* mutant mice died perinatally, as did the *Dvl2* or *Dvl3* knockout mice. The *NRX* mutant mice showed craniofacial defects, which may have caused their death by resulting in an inability to suckle. Knock-out mice harbouring gene deletions of components of the Wnt/β-catenin pathway (such as *β-catenin* (Wnt1-Cre-mediated) and *Axin2*) commonly display craniofacial defects [84,85]. In addition, we independently generated *NRX* mutant mice by a conventional gene targeting approach via homologous recombination. The above-mentioned craniofacial defects reported by Justice's group are all also observed in our *NRX* mutant mice. The fact that two *NRX* mutant mice strains, which were independently generated by different methods, show a very similar phenotype clearly indicates an indispensable role for NRX in normal murine development.

There are several NRX-related proteins in *C. elegans*. Three independent groups reported that the depletion of one of them, R05H5.3, via RNAi causes an embryonic lethal phenotype [86–88]. It should be noted that all NRX-related proteins in *C. elegans*, including R05H5.3, contain only one TryX-like domain and therefore more closely resemble TryX than NRX.

Future perspectives

Since the discovery of NRX as a physiological binding partner of Dvl, our understanding of this fascinating molecule at the molecular level has advanced substantially. In addition, analyses of the *NRX* mutant mice and *Xenopus* embryos have also revealed the essential function of NRX in multiple developmental stages. However, there still remain many unanswered questions. For example, what is the physiological relevance of H_2O_2-dependent activation of the Wnt/β-catenin pathway? Whilst we have speculated on several

possibilities in the previous section (e.g. that it may prevent unnecessary activation of apoptotic pathways or that it may be required for Wnt/β-catenin signalling itself), the answer remains to be experimentally determined.

Another unresolved issue is that of NRX involvement in tumourigenesis. It is well known that aberrant activation of the Wnt/β-catenin pathway leads to tumourigenesis and several genetic alterations were found in various components of the Wnt/β-catenin pathway. We showed that NRX-knockdown in culture cells results in an elevated growth rate and increases transformation potential in the presence of activated Ras or MEK [42]. So far, genetic alterations in the *NRX* locus have not been reported to occur in tumours. However, as mentioned in the previous section, some Wnt/β-catenin pathway components are epigenetically silenced in tumours. Also, it is known that tumour cells tend to have a higher level of ROS compared to normal cells [89]. While $NRX^{-/-}$ mice die perinatally, $NRX^{+/-}$ mice are apparently normal and fertile. However, modest but significant craniofacial abnormalities, which both we and Boles et al. [83] identified in $NRX^{-/-}$ mice, can still be observed in+/- littermates. Therefore, it will be of interest to observe these mice for a much longer period to determine whether $NRX^{+/-}$ mice display a high incidence of tumours.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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