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Human GW182 Paralogs are the Central Organizers for RNA-Mediated Control of Transcription

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Summary

In the cytoplasm, small RNAs can control mammalian translation by regulating the stability of mRNA. In the nucleus, small RNAs can also control transcription and splicing. The mechanisms for RNA-mediated nuclear regulation are not understood and remain controversial, hindering the effective application of nuclear RNAi and blinding investigation of its natural regulatory roles. Here we reveal that the human GW182 paralogs TNRC6A/B/C are central organizing factors critical to RNA-mediated transcriptional activation. Mass spectrometry of purified nuclear lysates followed by experimental validation demonstrates that TNRC6A interacts with proteins involved in protein degradation, RNAi, the CCR4-NOT complex, the mediator complex, and histone modifying complexes. Functional analysis implicates TNRC6A, NAT10, MED14, and WDR5 in RNA-mediated transcriptional activation. These findings describe protein complexes capable of bridging RNA-mediated sequence-specific recognition of noncoding RNA transcripts with the regulation of gene transcription.

eTOC Blurbs

Nuclear RNAi has the potential to add a previously unrecognized layer of control over mammalian gene expression. Hicks et al. use mass spectrometry to expand identification of protein partners that may play roles in RNA-mediated regulation of transcription and splicing.

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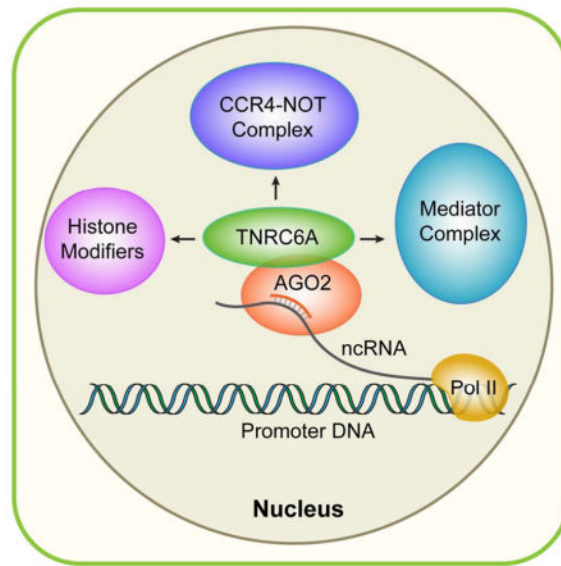
AUTHOR CONTRIBUTIONS

J.A.H, L.L., M.M., Y.C., O.V., AND K.C.J. executed experiments. J.A.H. helped prepare the manuscript. D.R.C supervised the experiments and wrote the paper.

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Introduction

MicroRNAs (miRNAs) are found in mammalian nuclei, as are key RNAi proteins like AGO2 and the GW182 paralogs TNRC6A, TNRC6B, and TNRC6C (Gagnon et al., 2014; Matsui et al., 2015). The presence of both small RNAs and RNAi factors in nuclei suggests that RNA-mediated recognition may regulate RNA-dependent processes like transcription or splicing. Despite its potential impact, nuclear RNAi has remained an unexplored facet of gene regulation.

There have been reports that miRNAs and duplex RNAs can affect gene transcription (Weinberg and Morris 2016; Kalantari et al., 2016a). While these reports have built a strong case for nuclear RNAi function, the detailed mechanism for transcriptional regulation has not been characterized, blocking progress towards understanding the broader significance of nuclear RNAi or critical unanswered questions regarding the roles it might play in normal physiology and development.

AGO2 is an essential cytoplasmic RNAi factor (Liu et al., 2004) that associates with small RNAs, assists recognition of complementary sequences, and induces cleavage of target RNAs when the match is fully complementary. In the nucleus, AGO2 is essential for RNA-mediated regulation of transcription (Chu et al., 2010) and splicing (Liu et al., 2012). Because of its established importance as an RNAi factor, we previously examined the potential for AGO2 to interact with proteins in cell nuclei. Mass spectrometry of the nuclear partners of AGO2, however, revealed only limited insights (Kalantari et al., 2016b). The GW182 paralogs TNRC6A, TNRC6B, and TNRC6C and the AGO variant AGO3 were the only partner proteins detected. The narrow range of proteins that interact with AGO2 was insufficient to explain the observed functional control of small RNAs on transcription.

We now examine the next shell of protein partners – those associated with TNRC6A. The GW182 family of proteins was first discovered as an autoantigen in patient serum (Estathioy

et al., 2002) and are characterized by glycine-tryptophan (GW) repeats within unstructured regions. TNRC6A and the closely related GW182 paralogs TNRC6B and TNRC6C are well known binding partners for cytoplasmic AGO2 (Yao et al., 2013; Pfaff et al., 2013). They stabilize AGO2-miRNA interactions and help localize AGO2 to cytoplasmic p-bodies.

TNRC6A is also found in cell nuclei (Gagnon et al., 2014) and may be involved in shuttling AGO2 into the nucleus (Nishi et al., 2013). TNRC6A is a multi-domain protein. Two domains play an essential role in silencing, the N-terminal GW-repeat domain and the bipartite silencing domain (Braun et al., 2013). These domains are embedded within unstructured regions containing multiple glycine-tryptophan (GW) repeats that can form scaffolds for protein complexes.

TNRC6 was chosen for proteomic analysis because of its demonstrated association with the critical RNAi factor AGO2, and its ability to be a scaffolding protein. TNRC6A, rather than TNRC6B or TNRC6C, was chosen for analysis because of the availability of an antibody suitable for isolating endogenous cellular protein from nuclear lysate for mass spectrometry (Fig. S1A). TNRC6A is also the most abundant paralog found in HeLa cells (Fig. S1B).

Here we report analysis of binding partners of TNRC6A in mammalian cell nuclei. We identify partners from multiple protein families, including mediator complex, histone modifiers, anaphase promoting complex, CCR4-NOT complex, and RNAi. Our data build a mechanistic framework for understanding the action of small RNAs and RNAi factors in mammalian cell nuclei and suggests that TNRC6 proteins are central organizing factors in this process.

RESULTS

Immunoprecipitation and Mass Spectrometry for TNRC6A

Nuclear extracts were prepared for large scale immunoprecipitations, typically requiring several hundred million cells. All samples were treated with either DNase I or Turbonuclease to digest chromosomal DNA prior to immunoprecipitation. Turbonuclease, a broad spectrum RNase and DNase, was used to evaluate the influence of RNA on protein interactions and those samples were termed “Turbonuclease-treated”. Samples treated with only DNase I were termed “DNase-treated”.

Nuclear extracts were free of the cytoplasmic marker protein GAPDH and the ER marker Calreticulin (Fig. S1C). After immunoprecipitation using anti-TNRC6A antibody and an anti-Rabbit IgG antibody as a control, the purified samples were analyzed by western blot to confirm the presence of TNRC6A (Fig. S1D). Because a high depth of coverage was desired, all samples were required to have a visible band corresponding to the molecular weight for TNRC6A (Fig. S1E).

Data was analyzed by semi-quantitative analysis using the normalized spectral index (SINQ) (Trudgian et al., 2011) (Tables S1–S2). SINQ provides spectral counts and ratios between sample and control for each protein. We required a minimum ratio of 4:1 for immunoprecipitated sample versus lysate control and at least six spectral counts be

observed. For turbonuclease-treated samples, a ratio cutoff of five was used to limit our protein list. The proteins with a ratio above four and below five can be found in Table S5. Experiments were performed in duplicate or triplicate and the standards for potential significance needed to be met in all replicates. We identified thirty-eight candidate proteins after turbonuclease treatment (Fig. 1A–C, Table S3) and thirty-three in samples treated with DNase I alone (i.e. no exposure to RNase activity) (Fig. 1D–F, Table S4). In many cases, more than one member of a protein complex was identified, bolstering the conclusion that the interactions might be physiologically significant. Most candidate proteins could be divided into three groups; RNAi factors, anaphase promoting complex, and proteins with known roles in transcription.

In addition to SINQ analysis, we performed stoichiometric analysis of protein interactions using the Intensity Based Quantification (iBAQ) method (Smits et al., 2013) (Fig. 2). Using this approach in our RNase-treated dataset, one protein, TJP1, was present at a 15:1 ratio versus TNRC6A—an unlikely outcome (Fig. 2A). TJP1 is not known to be a nuclear protein and, unlike all other candidate proteins, there is no obvious physiologic justification for it to associate with TNRC6A. The implausible ratio suggests this interaction is an artifact.

While stoichiometric ratios calculated by iBAQ should be considered estimates, it is interesting to note that The TNRC6A:AGO2 ratio was 1:2.2. This finding suggests that more than one AGO protein can bind simultaneously to TNRC6A (Fig. 2A–B). This finding would be consistent with the presence of numerous tryptophan residues on the surface of TNRC6A and their potential to interact with the surface of AGO2 (Fig. 2C). AGO3 was present at a 1:1 ratio (Fig. 2A–B). This finding is also consistent with our prior mass spectrometry using AGO2 as the bait protein that showed recovery of AGO3 (Kalantari et al. 2016b). Presumably, this previous result was mediated through the potential of AGO3 and AGO2 to bind simultaneously to TNRC6A. CNOT9 was identified at a 1:1 ratio, consistent with its known ability to bind TNRC6A (Fig. 2A–B) (Mathys et al., 2014). Almost all other candidate proteins were present at less than 1:1, reflecting either partial association or loss of interactions during purification. There may be multiple complexes, with some proteins involved in only a subset.

Verifying TNRC6A protein interactions

Representative proteins from key functional groups were chosen for closer analysis by immunoprecipitation in turbonuclease (RNase/DNase)-treated extracts (Fig. 3). We used immunoprecipitations with an anti-TNRC6A antibody elicited to a peptide sequence (575–625aa) within the TNRC6A protein that was not found in TNRC6B or TNRC6C. To cross-validate our findings, we also used antibodies elicited to bind the candidate proteins. The TNRC6A antibody binds the two isoforms of TNRC6A and that is verified via knockdown experiments (Fig. S2A). We also used a second anti-TNRC6A antibody, elicited to a different peptide (1225–1275aa) unique to TNRC6A, for immunoprecipitations and confirmed interactions (Fig. S2B).

AGO variants associate with nuclear TNRC6A

We identified interactions with AGO1, AGO2, and AGO3 in both sample sets. Regardless of whether RNase was added, AGO2 and AGO3 were among the proteins with the highest detected spectral counts and with high ratios for anti-TNRC6A samples versus controls (Fig. 1). The association between AGO proteins and TNRC6A is consistent with the well-known partnership between AGO and TNRC6A/B/C and our previous mass spectrometry using AGO2 as bait showing that the TNRC6 paralogs were the primary interacting partners for AGO2 in both cell cytoplasm and nucleus (Kalantari et al., 2016b). We had also previously used microscopy to demonstrate colocalization of AGO2 and TNRC6A (Gagnon et al., 2014).

Western analysis revealed that AGO2 was present in HeLa cell nuclei (Fig. 3A). Immunoprecipitation with anti-TNRC6A antibody pulled down AGO2 (Fig. 3B–F). Conversely, immunoprecipitation with anti-AGO2 antibody pulled down TNRC6A. Formation of an AGO2:TNRC6A complex is significant because AGO2 has the potential to mediate sequence-specific binding to RNA while TNRC6A has the potential to be a scaffold and recruit other protein factors.

TNRC6A associates with components of the anaphase promoting complex

In RNase-treated samples we identified interactions with components of the anaphase promoting complex (APC) (Fig. 1A–C). The APC is a ubiquitin ligase that contributes to several cellular pathways by controlling ubiquitin-mediated proteolysis (Chang and Barford, 2014). APC is a multi-protein complex and several subunits appeared to interact with TNRC6A including ANAPC1, 4, 5, and 7. Cell cycle proteins CDC 16, 23, and 27 associate with APC and were also detected. We only detected ANAPC4 in samples not treated with RNase (Fig. 1D–F), suggesting that the interaction between APC and TNRC6A may be more transient or involves RNA.

APC and associated proteins control ubiquitin mediated proteolysis during the cell cycle and its association with TNRC6A suggests a degradation pathway for nuclear RNAi factor complexes. ANAPC1, the core component of APC, was chosen for further study because an antibody suitable for western analysis was available. ANAPC1 is a predominantly nuclear protein in HeLa cells (Fig. 3A). Anti-TNRC6A antibody immunoprecipitated ANAPC1 while anti-AGO2 antibody did not (Fig. 3B). These data are consistent with TNRC6A occupying a bridging position between AGO2 and ANAPC1.

We also observed the interaction in nuclear extracts that were treated with DNase I only (Fig. S2C). This finding suggests the reason the interaction wasn't detected in extracts that had been treated with only DNase prior to mass spectrometry is because they might be more transient. The converse immunoprecipitation was not done because the available anti-ANAPC1 antibody was not adequate.

TNRC6A associates with proteins that modify histones

Our mass spectrometry using turbonuclease-treated samples also identified interactions between TNRC6A and several proteins known to modify histones (Fig. 1). The Mixed

Lineage Leukemia (MLL) complex is an H3K4 methyltransferase. We identified MLL complex subunits including MLL3 and MLL4 as well as associated factors including ASH2L, WDR5, RbB5, and AFF4. WDR5 is a member of several histone-modifying complexes and it is possible that its detection is not due to direct association with other MLL factors. We focused on WDR5 and RbBP5, both WD40 repeat proteins because they are the core proteins that interact to coordinate H3K4 tri-methylation activity (Dou et al., 2006).

An anti-TNRC6A antibody immunoprecipitated RbBP5 and an anti-RbBP5 antibody immunoprecipitated TNRC6A, but not AGO2 (Fig. 3C). However, an anti-AGO2 antibody also immunoprecipitated RbBP5 and we cannot exclude that the interaction is mediated through AGO2. When an anti-WDR5 antibody was used for immunoprecipitation we also detected interactions with TNRC6A but not AGO2 (Fig. 3C). We also observed an association between WDR5 and RbBP5.

We noted the potential for interactions with other proteins known to regulate transcription including histone acetylase NAT10, PCF11, GATAD2A, ZNF24 (Li et al. 2006), and SWI/SNF proteins ARID1A and SMARCD1. Based on antibody availability we focused on NAT10 and observed that the anti-NAT10 antibody immunoprecipitated TNRC6A, but not AGO2 (Fig. 3C). However, an anti-AGO2 antibody pulled out NAT10. We also observed interactions between NAT10, RbBP5, and WDR5 that had not been noted previously (Fig. 3C,G).

TNRC6A associates with members of the CCR4-NOT complex

The CCR4-NOT complex plays an important role in cytoplasmic RNAi by associating with TNRC6 proteins (Chekulaeva et al., 2011) and contributing to deadenylation and translational repression (Chen et al., 2014; Mathys et al., 2014). CCR4-NOT has also long been recognized to play a role in transcriptional regulation (Collart 2016). For example, the CCR4-NOT complex has been shown to promote activating histone marks, H3K4 tri-methylation and H3 and H4 acetylation (Mulder et al., 2007).

Our mass spectrometry of nuclear lysate after immunoprecipitation with anti-TNRC6A antibody identified multiple components of CCR4-NOT complex including CNOT1, CNOT3, CNOT7, CNOT9, and CNOT10. (Fig. 1). CNOT1 is a scaffolding protein that plays a central role in organizing the CCR4-NOT complex (Maillet et al., 2000). Both CNOT1 and CNOT9 (RQCD1) have been proposed to bind sites on the surface of TNRC6 protein (Fabian et al., 2011; Mathys et al., 2014), so we chose these proteins for further examination.

CNOT1 is predominantly expressed in HeLa cell cytoplasm but can also be readily detected in nuclei, while CNOT9 is primarily nuclear (Fig. 3A). Anti-TNRC6A antibody pulled down CNOT1 and anti-CNOT1 antibody pulled down TNRC6A (Fig. 3D). Anti-AGO2 antibody pulled down CNOT1, but not CNOT9 (Fig. 3D, Fig. S2D). Immunoprecipitation with anti-CNOT9 antibody recovered TNRC6A but not AGO2 (Fig. 3E).

TNRC6A associates with members of the mediator complex

The mediator complex is composed of twenty-one proteins, is essential for controlling transcription by RNA polymerase II and can participate in either positive or negative regulation (Ansari and Morse, 2013). Regardless of whether turbonuclease was present in our samples, we observed the interaction of TNRC6A and several different mediator subunits including MED12, MED13, MED1, and MED14 (Fig. 1). An interaction with MED27 was observed only after treatment with turbonuclease, and MED4, MED6, MED 17, and MED23 were observed in samples treated only with DNase I. Those interactions may be more transient.

MED14 was chosen as a representative subunit for further analysis for three reasons: 1) it was recently identified as both a structural and functional backbone of the mediator complex (Cevher et al., 2014); 2) it was identified regardless of treatment with turbonuclease; and 3) the anti-MED14 antibody was the most effective anti-mediator antibody available to us. Western analysis confirmed the nuclear localization of MED14 in HeLa cells (Fig. 3A).

The anti-MED14 antibody pulled out TNRC6A but not AGO2 (Fig. 3F). Anti-TNRC6A antibody pulled out MED14, while anti-AGO2 antibody did not. These data suggest that MED14 has a closer association with TNRC6A than AGO2. The anti-MED14 antibody also pulled down CNOT1 and ASH2L, suggesting the potential for additional interactions with proteins that regulate transcription.

Association of candidate proteins after fractionation

To further characterize nuclear complexes containing RNAi proteins we used size exclusion chromatography followed by anion exchange chromatography to separate candidate proteins by size and charge (Fig. 4). The samples had not been treated with turbonuclease, increasing the potential for RNA-mediated interactions. Size exclusion chromatography of the nuclear extract revealed that TNRC6A was primarily identified in two fractions (Fig. 4A). These fractions also contained AGO2, CNOT1, CNOT9, ANAPC1, NAT10, RbBP5, WDR5, MED14, and Histone H3. It should be noted that AGO2 is found in some fractions that do not contain TNRC6A, suggesting the possibility that it forms complexes independent of TNRC6A. Alternatively however, this result might be due to the AGO2-TNRC6A complex dissociating during purification and chromatography.

Size exclusion fraction C containing TNRC6A was then applied to a Mono-Q anion exchange column. TNRC6A was found primarily in two fractions (Fig. 4B). These fractions also contained CNOT1, CNOT9, NAT10, RbBP5, WDR5, and MED14. While histone H3 had co-purified with TNRC6A after size exclusion, only a minimal amount co-purified after anion exchange. These data from sequential purifications are consistent with the conclusion that TNRC6A exists in complex with the proteins identified as candidate proteins by mass spectrometry and reciprocal immunoprecipitations. We had previously observed co-purification of nuclear AGO2 and TNRC6A after chromatography (Gagnon et al., 2014).

Other proteins implicated by mass spectrometry

Mass spectrometry suggested interactions between TNRC6A and two paraspeckle proteins, SFPQ and PSPC1. Paraspeckles are nuclear RNA bodies of unknown function that can affect protein localization and association with the RNA NEAT1 (Nakagawa and Hirose, 2012). SFPQ and PSPC1 were among the most strongly detected proteins obtained from samples that had been treated with DNase only (Fig. 1D–F). They were not detected in samples treated with turbonuclease (Fig. 1A–C), suggesting that their inclusion in complexes requires RNA. These data suggest that nuclear RNAi factors at least partially reside in paraspeckles.

We also observed four proteins involved in DNA repair, TP53BP1, CCAR2, and TNKS1BP1, and MDC1 in samples treated with DNase I (Fig. 1A–C). This finding is consistent with reports that RNAi factors can bind RNA during the DNA damage response (Wei et al., 2012). Stoichiometric analysis indicated TP53BP1 and CCAR2 had approximately a 1:1.52 and 1:0.88 ratio, respectively, reinforcing the suggestion that these are good candidates for follow-up study (Fig. 2).

Functional analysis: Impact on RNA-mediated transcriptional activation

Immunoprecipitation confirmed the association between TNRC6A and highly ranked candidate proteins identified from mass spectrometry. To investigate the functional involvement of candidate proteins we tested their involvement in RNA-mediated activation of cyclooxygenase 2 (*COX-2*). *COX-2* was chosen because transcriptional activation can be triggered by miR-589 and RNA12nc. miR-589 is a miRNA with two partially complementary binding sites within a noncoding transcript that overlaps the *COX-2* promoter (Matsui et al., 2013). RNA12nc is a “miR-like” duplex with central mismatches designed to disable the cleavage activity of AGO2 but has overall complementarity to a noncoding transcript that overlaps the *COX-2* promoter.

The levels of activation are robust, specific (sensitive to just one mismatch within the RNA duplex) and easily detectable (> 20-fold) making *COX-2* a sensitive system for examining expression (Matsui et al., 2013). The previous experiments had demonstrated that AGO2 was required for this process and addition of promoter-targeted RNA led to increased recruitment of RNA polymerase, increased expression of pre-mRNA, mRNA, and protein, and increased activating histone marks (H3K4me3 and H4Ac). As little as one mismatch in the seed sequence of the guide strand of a duplex RNA was sufficient to ablate RNA-mediated activation of gene expression. Antisense oligonucleotides designed to reduce expression of the noncoding RNA target strand also blocked RNA-mediated activation of gene expression.

To determine involvement of the candidate proteins, our first step was to transfect siRNAs into A549 lung cancer cells to block expression of a candidate gene. In a second transfection, we introduced promoter-targeted duplex RNA12nc known to activate expression of *COX-2* protein or mRNA (Matsui et al., 2013). For all experiments, we also introduced control duplex RNA, siGL2, that lacks complementarity to genes within the human genome and does not activate *COX-2* expression.

When we added the duplex RNA complementary to the *COX-2* promoter to A549 cells we observed enhanced *COX-2* expression (Fig. 5). When all TNRC6 paralogs were inhibited, activation was not observed (Fig. 5A, Fig. S3A), confirming functional involvement of TNRC6 in RNA-mediated gene activation. Inhibiting just one TNRC6 paralog was not adequate, suggesting overlapping contributions (Matsui et al., unpublished). We had previously observed that inhibiting AGO2 also reversed *COX-2* activation by RNA12nc (Matsui et al., 2013).

We next examined roles for proteins associated with the regulation of transcription. Inhibiting expression of WDR5 reversed RNA-mediated activation of *COX-2* expression (Fig. 5B, Fig. S3B) (Matsui et al., 2013). Inhibiting expression of NAT10 (Fig. 5C, Fig. S3C) or MED14 (Fig. 5D, Fig. S3D) also reversed RNA-mediated gene activation. These data are consistent with the immunoprecipitation data demonstrating association of WDR5, NAT10, and MED14 with TNRC6A (Fig. 2). Chromatin immunoprecipitation demonstrated increased recruitment of WDR5 to the *COX-2* promoter after addition of activating RNA (Fig. S4).

By contrast to the reversal of gene expression after knocking down candidate proteins WDR5, NAT10, and MED14, inhibiting expression of the CCR4-NOT complex members CNOT1 (Fig. 5E, Fig. S3E) or CNOT9 (Fig. 5F, Fig. S3F) increased levels of *COX-2* protein above what they would be from addition of activating duplex RNA alone. These data suggest that the CCR4-NOT complex is not required for RNA mediated transcriptional activation of *COX-2*. The CCR4-NOT complex is known to play a role in RNA-mediated translational silencing (Chekulaeva et al., 2011). The increase of *COX-2* protein levels that we observe may be related to the removal of regulation by a miRNA.

We also examined the functional role of the anaphase promoting complex component ANAPC1 (Fig. 5G, Fig. S3G). When expression of the anaphase promoting complex subunit ANAPC1 was knocked down and RNA12nc added, expression of *COX-2* protein increased above the level produced by introducing the activating RNA alone. This result is consistent with the conclusion that anaphase promoting complex contributes to degradation of RNAi factors.

DISCUSSION

Nuclear RNAi

The demonstrated ability of small RNAs to control splicing and transcription through nuclear RNAi in mammalian cells (Kalantari et al. 2016; Weinberg and Morris 2016) suggests an unanticipated layer of biological regulation that goes beyond the control exerted by textbook protein transcription and splicing factors. For transcriptional control, nuclear RNAi is believed to function through recognition of nascent transcripts rather than direct binding to chromosomal DNA. The central mechanistic question, therefore, has been how recognition of an RNA transcript can trigger a change in mRNA synthesis at a gene promoter. Defining the action of miRNAs, however, is not simple even for the miRNAs that function through post-transcriptional silencing in the cytoplasm – a process that has been the

focus of much more research. Our goal was to use mass spectrometry to build a better model of the proteins recruited to participate in nuclear RNAi.

Previous lessons from RNA control of COX-2 activation

We used RNA-mediated activation of COX-2 expression as a reporter of function because aspects of the mechanism had been characterized in detail (Matsui et al., 2013). Duplex RNAs complementary to the COX-2 promoter activate expression of COX-2 pre-mRNA, mRNA, and protein. Activation requires seed sequence complementarity to transcript overlapping the gene promoter, expression of AGO2, and expression of TNRC6. This prior work suggested that introduction of a promoter-targeted small RNA into cells could lead to binding of an AGO2-TNRC6-small RNA complex to a promoter transcript, enhanced binding of RNA polymerase, and dramatically upregulated levels of COX-2 expression. Unanswered questions included the identity of protein partners and the role of TNRC6.

TNRC6 is the central organizing factor controlling nuclear RNAi

In stark contrast to the narrow group of interacting partners for AGO2 – the three TNRC6 paralogs and AGO3 (Kalantari et al., 2016b) - we discovered many candidate partners for TNRC6A (Fig. 1). The finding that TNRC6A interacts detectably with more proteins is consistent with the known ability of GW182 family members to act as scaffolding proteins (Yao et al., 2013; Pfaff et al., 2013). Detection of proteins by mass spectrometry will become weaker as interactions become more indirect and a scaffolding protein is likely to bind more proteins directly. While mass spectrometry using AGO2 as bait may readily detect GW182 family members (which bind directly to AGO2), the proteins that directly bind the scaffolding protein GW182 may not be detected as candidates for association with AGO2 because those associations are indirect and more likely to be lost during purification.

Most of the proteins identified in our TNRC6A datasets clustered in six well-defined functional groups. These included RNAi factors (AGO1, AGO2, AGO3), members of the APC (ANAPC1, ANAPC4, ANAPC5, ANAPC7, ANAPC16, CDC16, CDC23, CDC27), members of the mediator complex (MED1, MED4, MED5, MED12, MED13, MED14, MED17, MED23, MED27), members of histone modifying complexes (MLL3, MLL4, WDR5, ASH2L, RbBP5, NCOA6, NAT10), members of the CCR4-NOT complex (CNOT1, CNOT3, CNOT7, CNOT9, CNOT10, CNOT11). The finding that interacting partners cluster into groups that define known protein complexes reinforces the likelihood that the mass spectrometry is efficiently identifying legitimate protein partners. Furthermore, our data identify known interacting partners of TNRC6A—AGO proteins and members of the CCR4-NOT complex. While the interaction with CCR4-NOT in the nucleus has not been reported previously, in retrospect it might have been expected due to the nuclear presence of both the CCR4-NOT complex and TNRC6A.

Our mass spectrometry and immunoprecipitation data, along with the known role of GW182 family members as protein scaffolds, suggest that TNRC6A is a physical bridge between AGO2, the protein responsible for binding guide strand RNA and facilitating recognition of cell RNA targets, and proteins that affect transcription (Fig. 3G, 5H). TNRC6A (and likely its TRNC6B/C paralogs), not AGO2, appears to be the central organizing factor bringing

together AGO2, CCR4-NOT subunits, mediator proteins, and histone modifiers (Fig. 3G). In addition, all proteins verified by co-immunoprecipitations eluted together in the same fractions after two chromatographic separations (Fig. 4). This further supports the conclusion that these proteins are in complexes together.

Experimental caveats

We note several caveats to our conclusions. 1) Some members of AGO2/GW182 complexes may not be ranked as “detectable” under the conditions and candidate identification parameters used. Failure to definitively identify an interaction is not proof that the interaction (direct or indirect) does not occur. We did not use protein crosslinking and our approach was designed to emphasize detection of high priority candidates rather than being an exhaustive listing of all plausible partners; 2) The exact geometry, composition of protein partners, and stoichiometry of complex formation remains unknown. Our model (Figure 5H) for how proteins can be arranged is only one possible arrangement and it is possible binding occurs in a stepwise fashion. Many possible protein complexes focused around the TNRC6:AGO partnership are possible; 3) While the partnership of the TNRC6 paralogs and AGO2 is well established, it is possible that the TNRC6 paralogs play roles that are independent of AGO2 and that AGO2 plays roles that are independent of TNRC6A. Independence of AGO2 from TNRC6 and vice versa will depend on their relative stoichiometry in the cytoplasm and nucleus; 4) Our functional analysis focused on COX-2 and does not address how sequence-specific RNA-AGO2-TNRC6 protein complexes might affect other genes or how the composition of the complexes may change from one gene to the next; and 5) While RNA-mediated control of gene expression can be a robust phenomenon, the extent to which it plays a role in endogenous gene regulatory pathways remains unclear and is highest priority question for future research.

Conclusion

Nuclear RNAi, regardless of whether it is controlling splicing, transcription, or some other nuclear process, would have distinct advantages as a mechanism for evolution because it would permit sequence specific control of gene expression by miRNAs. The evolution of a miRNA to control transcription of a specific gene would likely be more straightforward than evolution of a protein. Synthetic RNAs have proven to be robust regulators of gene expression in nuclei and it seems reasonable to hypothesize that that evolutionary pressure should lead cells to use the mechanism. This study identifies proteins that may take part in protein complexes that associate with RNAi factors in cell nuclei to control transcription in conjunction with small RNAs. Many of these protein partners are involved in RNA transcription and provide obvious potential bridges between RNA recognition and the control of gene transcription.

EXPERIMENTAL PROCEDURES

Extract Preparation

Cytoplasmic extract was collected by lysing cells with Hypotonic Lysis (HL) Buffer (10mM Tris, pH 7.4, 10mM NaCl, 3mM MgCl₂, 0.3% NP-40) at 1mL/150mm dish. The nuclear pellet was spun down at 500xg for 5 minutes. The supernatant was saved as the cytoplasmic

extract and 140mM KCl and 10% glycerol were added. The nuclear pellet was washed with 10mL cold Hypotonic Lysis buffer 3X for 5 minutes. Nuclear extract was collected by adding Nuclear Lysis (NL) Buffer (20mM Tris, pH 7.4, 150mM KCl, 3mM MgCl₂, 0.5% NP-40, 10% Glycerol) at a concentration of 0.5 mL/150mm dish. A 27-gauge needle was used to lyse the nuclei in addition to NL buffer. Nuclear extracts were treated with either Turbonuclease or DNase I to digest chromatin. While TNRC6A knockout cells would also provide a useful reagent for comparisons, it was not straightforward to provide these due to the complications of chromosome duplications in HeLa cells.

Western analysis and transfections

Western blots to determine the purity of nuclear and cytoplasmic fractions from TurboNuclease-treated HeLa cells as described (Gagnon et al., 2014). Co-immunoprecipitations and western analysis were performed as described (Kalantari et al., 2016). Double transfection experiments were performed as described (Matsui et al., 2013).

Mass Spectrometry and data analysis

Sample preparation, immunoprecipitation with an anti-TNRC6A antibody (Bethyl A302–329A), and mass spectrometry analysis was performed as described previously (Kalantari et al., 2016). Turbonuclease-treated samples were run on the Orbitrap Elite (OE) and DNaseI-treated samples were run on the Orbitrap Fusion Lumos (OFL).

Chromatographic Separations

Chromatographic separations were performed as described (Gagnon et al., 2014) in HeLa cell nuclear extract treated with SUPERase-In. Western blot analysis was performed as described above.

Statistical methods

For qPCR data analysis for the TNRC6 paralog expression levels, the relative expression level for each gene was calculated based on the average of the two primer sets for each gene, with 6 replicates for each primer set. P values were calculated from student's t tests using relative mRNA levels of each paralog. For ChIP-qPCR, p values were calculated using student's t tests relative to mismatch control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- TNRC6 is the central organizing factor for nuclear RNAi
- Interactions with mediator, CCR4-NOT, anaphase promoting complex, histone modifiers
- TNRC6, MED14, NAT10, and WDR5 are essential for RNA-mediated gene activation
- Biochemical connection between transcriptional control and nuclear RNAi

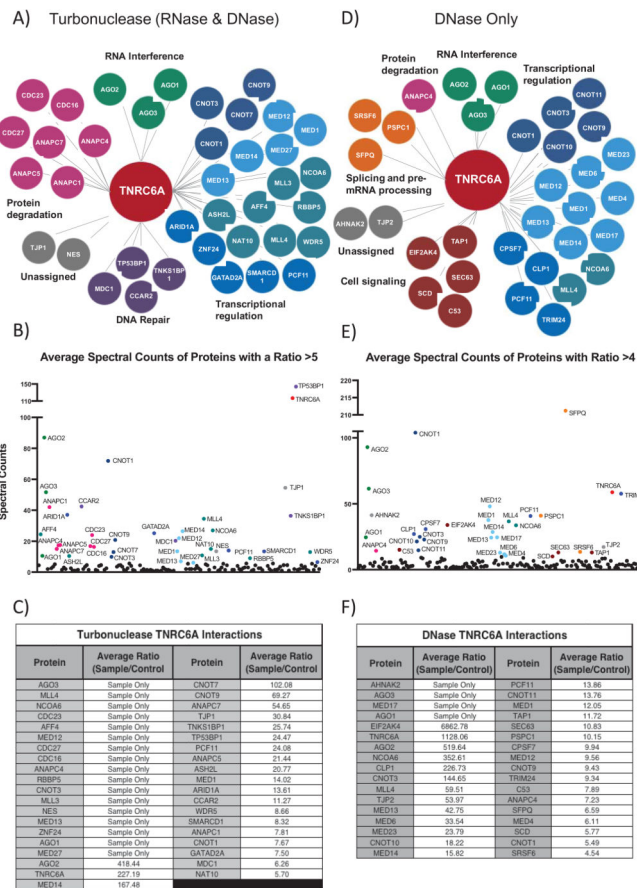


Figure 1. Identification of candidate protein partners for TNRC6A

Data was obtained after immunoprecipitation with anti-TNRC6A antibody and mass spectrometry. For parts (A), (B), and (C), samples were treated with turbonuclease, a broad-spectrum nuclease that has both RNase and DNase activity. (A) Bubble plot of candidates. (B) Scatterplot of spectral counts of all proteins with ratios >5 (cutoff). The significant proteins are colored corresponding to their category colors in (A). (C) Sample/control ratios of significant candidate proteins. For parts (D), (E), and (F), turbonuclease was not used to digest RNA, but DNase I was used to digest chromatin. (D) Bubble plot of candidates. (E) Scatterplot of spectral counts of all proteins with ratios >4 (cutoff). The significant proteins are colored corresponding to their category colors in (D). (F) Sample/control ratios of significant candidate proteins. Mass spectrometry for turbonuclease-treated samples was performed in duplicate and in triplicate for samples treated with DNase I. Complete experimental data is provided in the Tables S1 and S2. Candidate proteins for turbonuclease datasets that had ratios between 4 and 5 are listed in Table S5.

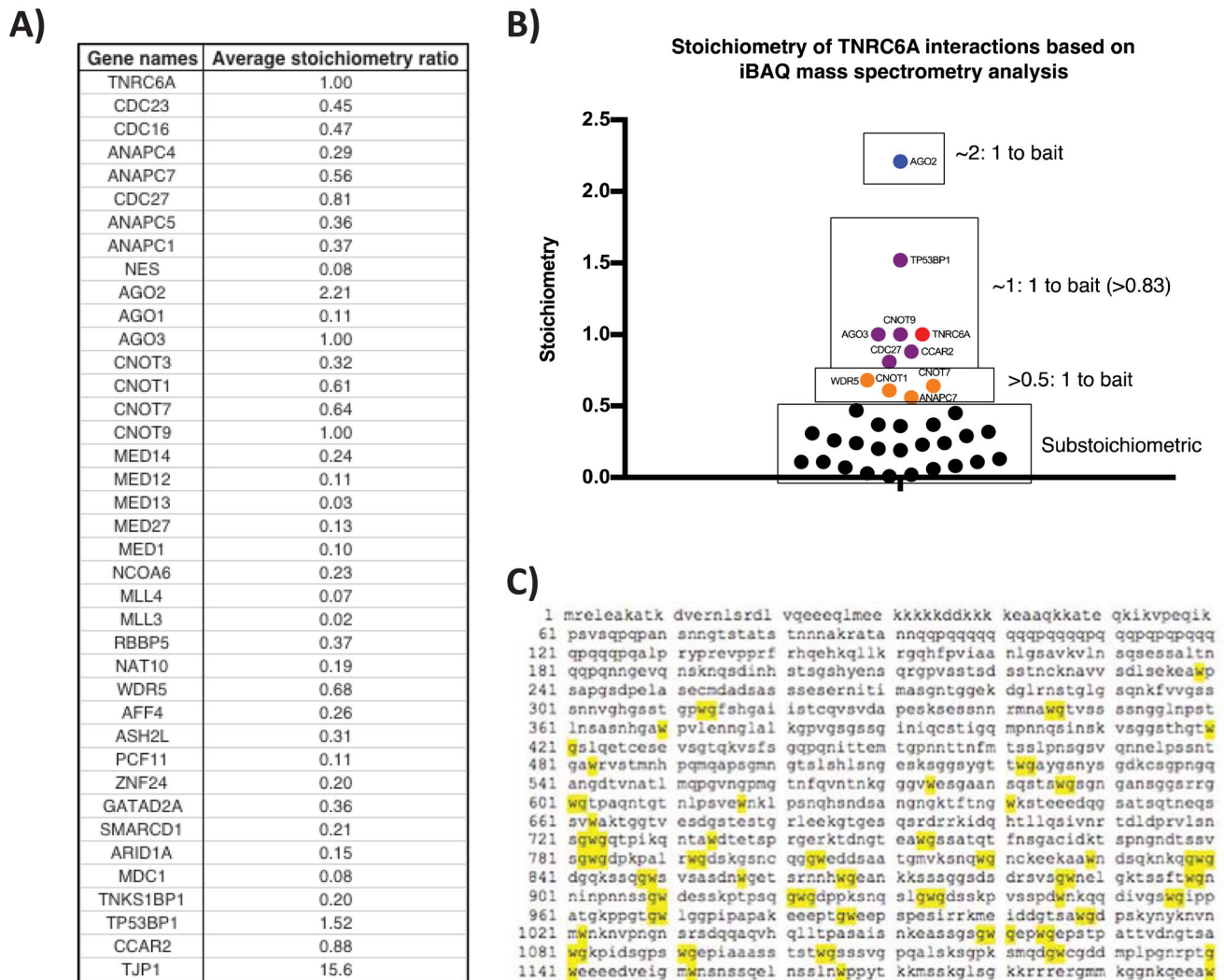


Figure 2. Stoichiometry analysis of mass spec samples with iBAQ values

(A) Stoichiometry values relative to TNRC6A (=1) levels. All iBAQ values for control (IgG) were subtracted from sample values. Turbonuclease samples were used in this case. (B) Histogram representing the stoichiometry ratios. TJP1 (=15.6) is excluded from this graph. (C) TNRC6A (isoform 1) N terminal AGO binding domain. Tryptophan (W) and Glycine-Tryptophan (GW) repeats are highlighted to indicate potential AGO binding sites. There are more GW repeats within the C terminal domain (not shown).

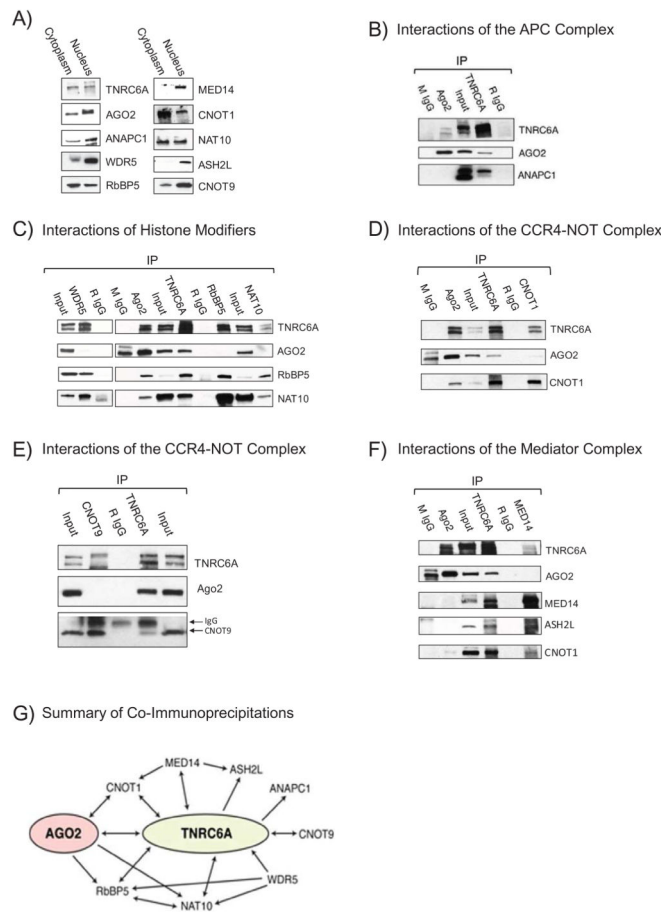


Figure 3. Validating the interactions of candidate proteins in Turbonuclease-treated nuclear extracts

Western analysis of candidate proteins after immunoprecipitations. These immunoprecipitations were performed in nuclear extracts that had been treated with turbonuclease to degrade RNA and chromatin. (A) Distribution of candidate proteins between cytoplasm and purified nuclei. Co-immunoprecipitations representing (B) Interactions of ANAPC1, a member of the APC, (C) Interactions of histone modifier proteins WDR5, RbBP5, and NAT10, (D,E) Interactions CCR4-NOT proteins CNOT1 and CNOT9, (F) Interactions of MED14, a member of the mediator complex, and (G) Summary of Co-immunoprecipitation experiments. Fractionation controls can be found in Fig. S1C. Validation of both TNRC6A isoforms as seen in western blots can be found in Fig. S3A and may not always show distinct bands in gels that are run for a shorter amount of time. The bands in the Mouse IgG lane present in the AGO2 blots in C,D, and F are not the size of AGO2 and are caused by impurities in the Mouse IgG.

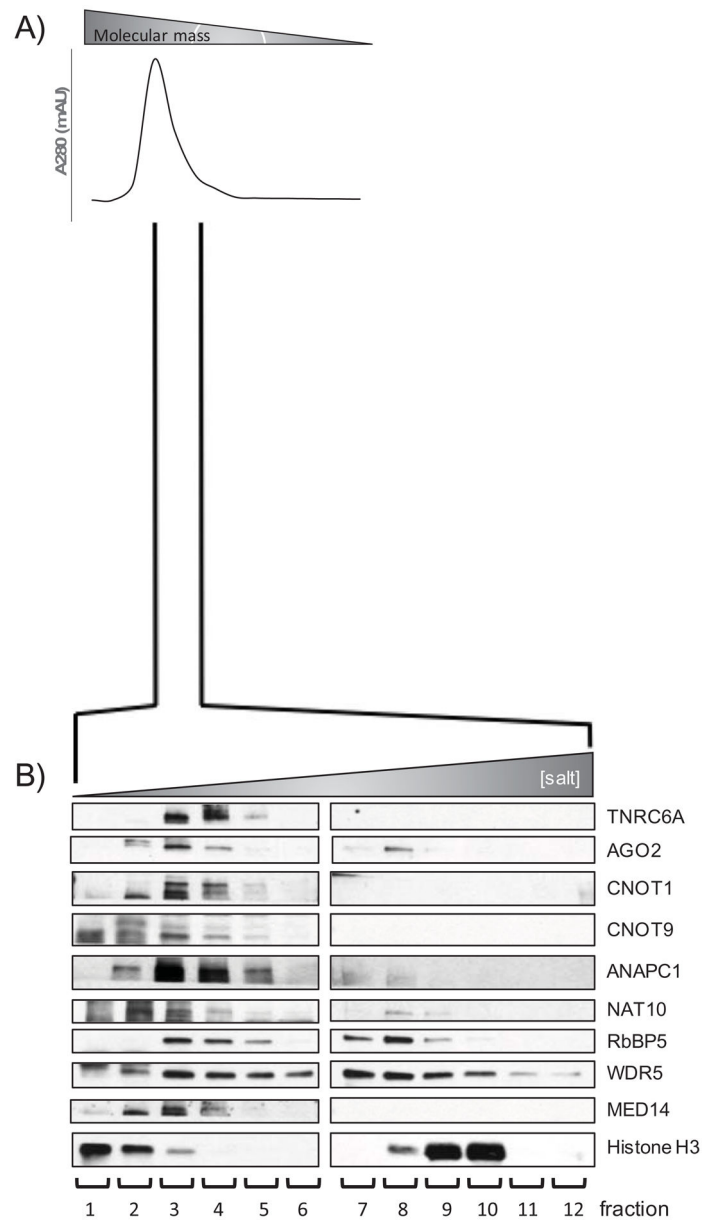


Figure 4. Sequential size exclusion and anion exchange chromatography to detect potential complexes with TNRC6A

All FPLC was performed with nuclear extracts not treated with RNase. (A) Size exclusion chromatography fractions with decreasing size. Fraction C was used for subsequent fractionations. (B) Anion exchange chromatography of size exclusion fraction C with increasing salt concentration. Western blots were performed in each fraction for candidate proteins.

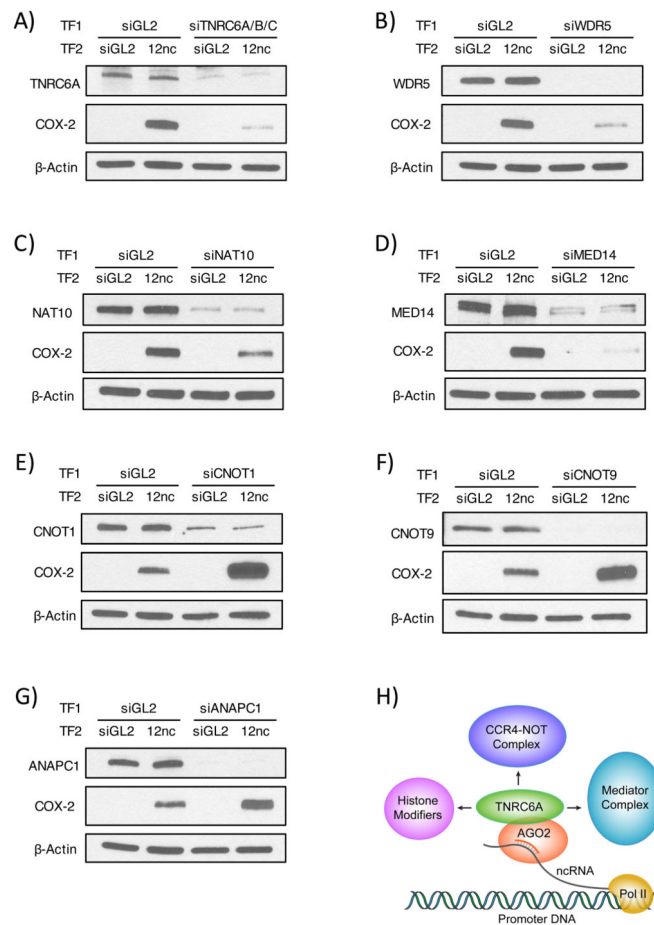


Figure 5. Functional roles for candidate proteins during RNA-mediated activation of COX-2 expression

Western analysis of COX-2 protein expression in presence or after depletion of candidate proteins followed by treatment with siGL2 or RNA12nc. Effect of using duplex RNAs to deplete (A) TNRC6A/B/C, (B) WDR5, (C) NAT10, (D) MED14, (E) CNOT1, (F) CNOT9, and (G) ANAPC1. 25 nM duplex siRNA was used in all experiments. (H) Summary of TNRC6A interactions. siGL2 is a control duplex RNA with minimal complementarity to other sequences within the human genome. TF1 = added during first transfection. TF2 = added during second transfection.