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N-Myc is a downstream target of RET signaling and is required for transcriptional regulation of $p18^{Ink4c}$ by the transforming mutant RET^{C634R}

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ABSTRACT

Inherited activating mutations in RET predispose humans to Multiple Endocrine Neoplasia type-2 (MEN2). The MEN2A-specific mutation RET^{C634R}, RET2A, has been shown to simultaneously downregulate the CDKIs p18 and p27, and upregulate cyclin D1. Importantly, the loss of p18 is necessary and sufficient for RET2A-mediated hyperproliferation. The loss of N-Myc in mice results in embryonic lethality due to a lack of neuronal progenitor cells that fail to proliferate, correlate with accumulation of p18 and p27. Therefore, N-Myc may regulate expression of both CDKIs. Also, N-Myc is expressed predominantly in neuroendocrine cells that give rise to the primary cell types affected in MEN2A. Together these studies suggest that N-Myc is a downstream target of RET2A signaling that prevents accumulation of p18 and/or p27. We report that MAPK activation by RET2A leads to a transient induction of N-Myc mRNA and protein levels, and that N-Myc induction is required to maintain low p18 and p27 levels. Induced N-Myc levels correlate with increased binding of N-Myc to an *initiator* consensus binding site in the p18 promoter, and this binding is essential for RET2A-mediated transcriptional regulation of p18. Finally, loss of N-Myc induction prevents RET2A-mediated hyperproliferation. Our results demonstrate for the first time that N-Myc is a downstream target of RET2A signaling, and propose that induction of N-Myc by RET2A is a key step leading to lower p18 levels during MEN2A tumorigenesis.

1. Introduction

The mammalian mitotic cell cycle is divided into four distinct phases, G_1, S, G_2 and M (Sherr, 1994). Progression through the cell cycle is regulated by the activation and subsequent inactivation of a family of serine/threonine kinases known as Cyclin Dependent Kinases (CDKs) (Sherr and Roberts, 1999). In the G_1 phase, positive and negative growth signals regulate CDK activity through numerous mechanisms such as changes in the expression patterns of cyclins and CDK

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Abbreviations: MEN2, multiple endocrine neoplasia type-2; CDK, cyclin dependent kinase; CDKI, CDK inhibitor; PTC, papillary thyroid carcinoma; MTC, medullary thyroid carcinoma; PC, pheochromocytoma; TF, transcription factor; *inr*, *initiator*; NPC, neuronal progenitor cells; GNP, granule neuron progenitor; qPCR, quantitative polymerase chain reaction.

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Inhibitors (CDKIs) (Sherr, 1993). Association of a CDK with a positively regulating Cyclin D or E subunit leads to its activation, whereas binding with CDKIs allows for negative CDK regulation (Sherr and Roberts, 1999). Based on binding properties, the CDKIs are divided into two families, the Inhibitor of Kinase 4 (Ink4: $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$) and CDK/Kinase Inhibitory Proteins (Cip/Kip: $p21^{Cip1}$, $p27^{Kip1}$ and p57).

Intuitively, overexpression of positively regulating cyclins or loss of negatively regulating CDKIs should lead to uncontrolled growth and tumorigenesis (Sherr, 1996). Indeed, in mice, loss of both $p18^{Ink4c}$ and $p27^{Kip1}$ (hereafter referred to as p18 and p27 respectively) leads to a tumor phenotype of primarily endocrine organs including the pituitary, pancreas, thyroid, parathyroid and adrenal glands (Franklin et al., 1998, 2000). This multiple tumor phenotype mimics the tumor spectrum seen in human Multiple Endocrine Neoplasia type-1 and type-2 combined (MEN1 and MEN2). This suggests that p18 and p27 are regulated by the protein products of *MEN1* and *RET,* mutations in which, are linked to MEN1 and MEN2 syndromes respectively.

RET is a proto-oncogene encoding a transmembrane receptor tyrosine kinase that is activated by binding to its ligands and co-receptors (Kodama et al., 2005). Constitutively activating mutations in RET, e.g. the RET/PTC translocation found in the Papillary Thyroid Carcinoma (PTC) patient-derived RET/PTC cell line, results in constitutive oncogenic activation of RET and leads to a MAPK pathway-mediated decrease of p27 (Vitagliano et al., 2004). We have previously shown that expression of another constitutively active mutant, RET^{C634R} (RET2A), leads to increased MAPK activation and decreased p18 and p27 levels which correlate with increased proliferation (Joshi et al., 2007). We also showed that low p18 levels are critical for RET2A-mediated hyperproliferation which underscores the importance of p18 regulation in RET2A-mediated oncogenesis. In patient-derived medullary thyroid carcinoma (MTC) and pheochromocytoma (PC) samples, only 13.8% (4 of 29 MTC and PC combined) tumors harbor somatic inactivating mutations in *p18* (van Veelen et al., 2009). Furthermore, in all 4 tumors, the inactivating *p18* mutations are in a heterozygous state and coincide with a germline *RET2A* mutation, indicating that the remaining wildtype *p18* allele in the tumors may be epigenetically downregulated by oncogenic RET.

The mechanism of p18 regulation is complex and remains largely unexplored. Phelps et al. (1998) have reported that p18 is differentially regulated at both transcriptional and translational levels in C2C12 murine myoblasts. It is transcribed as two distinct mRNA species, a short 1.2 kb p18S and a long 2.4 kb p18L transcript. Although it is known that only one transcript predominates, relevant promoters and regulatory elements remain ill defined. Translationally, only p18S encodes p18 protein (Phelps et al., 1998). Using various human breast cancer cell lines, Blais et al. (2002) showed that E2F1 and sp1 cooperatively enhance p18L transcription however, they were unable to detect the p18S transcript in their model system. In luminal A breast tumors, Pei et al. (2009) show an inverse correlation in GATA3 and p18 expression and increased GATA3 binding to consensus binding sites in the p18 promoter region. However, direct transcriptional response in terms of p18S or p18L transcript levels was not assessed. We have previously reported that expression of RET2A leads to decreased levels of the p18S transcript but not p18L (Joshi et al., 2007). Therefore in order to understand RET2A-mediated effects on p18S transcription, in this study, we carried out detailed analysis of the putative p18S promoter. Specifically, we searched for a *bona fide* target transcription factor of RET2A signaling, which could prevent accumulation of p18S mRNA. We report for the first time that the transcription factor N-Myc is a target of RET2A signaling and that it is induced in a MAPK dependent manner by RET2A. Increased binding of N-Myc to an *initiator* (*inr*) consensus binding site within the p18S promoter in response to RET2A expression leads to maintenance of low p18S mRNA (and protein) levels, correlate with increased proliferation. Lastly, we show that the RET2A-mediated transient induction of N-Myc is required for its effects on p18S transcription and hyperproliferation.

2. Results

2.1. Search for a putative transcription factor target of RET2A that mediates p18 transcriptional regulation

To understand how RET2A regulates p18S transcription, we searched for putative transcription factor (TF) targets of RET2A signaling that could bind to the p18 promoter. A detailed search for highly conserved cis-acting elements across human, mouse and rat genomes in a 5500 basepair region upstream of the p18 translation start site (TSS, set to +1) uncovered the presence of several consensus TF binding sites (method presented in Supplementary methods and data shown in Supplementary Table 1). We used three criteria that would qualify a TF as a likely candidate for RET2A-mediated p18 transcriptional regulation. First, we predicted that a *bona fide* downstream target of RET2A signaling capable of regulating p18S transcription would be expressed in adrenal chromaffin and thyroid parafollicular C cells or in endocrine organs. Developmentally, adrenal chromaffin and thyroid parafollicular C cells derive from Neuronal Progenitor Cells (NPCs) and it is known that during murine development, N-Myc is expressed exclusively in NPCs and other neuroendocrine cells (Downs et al., 1989). Second, N-Myc is regulated by the MAPK pathway which is required for p18 regulation by RET2A. Treatment of KP-N-RT human neuroblastoma cells with IGF-I results in a MAPK dependent N-Myc induction (Misawa et al., 2000). Thirdly, in developing cerebella, loss of N-Myc results in accumulation of p18 and p27, correlate with growth arrest in cerebellar Granule Neuron Progenitors (GNPs) (Zindy et al., 2006). In early neural development, expression of N-Myc in NPCs is required to maintain low p18 and p27 levels correlate with rapid NPC proliferation (Knoepfler et al., 2002). Loss of both p18 and p27 expression in an N-Myc null background restores normal proliferation of the GNPs, suggesting that during embryonic development, both p18 and p27 are regulated by N-Myc to promote proliferation. Taken together, these studies make N-Myc a likely candidate to study as a downstream intermediate in RET2A/MAPK-dependent p18 transcriptional repression. N-Myc has been shown to act as a transcriptional inhibitor by binding to an *inr* consensus sequence, although the commonly accepted role of N-Myc is enhancer box (E-box)–mediated