Rac-1 and Raf-1 kinases, components of distinct signaling pathways, activate myotonic dystrophy protein kinase

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Abstract Myotonic dystrophy protein kinase (DMPK) is a serine–threonine protein kinase encoded by the myotonic dystrophy (DM) locus on human chromosome 19q13.3. It is a close relative of other kinases that interact with members of the Rho family of small GTPases. We show here that the actin cytoskeleton-linked GTPase Rac-1 binds to DMPK, and coexpression of Rac-1 and DMPK activates its transphosphorylation activity in a GTP-sensitive manner. DMPK can also bind Raf-1 kinase, the Ras-activated molecule of the MAP kinase pathway. Purified Raf-1 kinase phosphorylates and activates DMPK. The interaction of DMPK with these distinct signals suggests that it may play a role as a nexus for cross-talk between their respective pathways and may partially explain the remarkable pleiotropy of DM.

Key words: Myotonic dystrophy protein kinase; Rac-1; Raf-1; Rho-type GTPases

1. Introduction

Myotonic dystrophy protein kinase (DMPK) is the defining member of the 'myotonic dystrophy family of protein kinases' (MDFPK) [1] because it is the protein product of the locus mutated in myotonic dystrophy (DM) [2,3]. Other putative serine–threonine protein kinases belonging to this group are *Neurospora* Cot1, *Drosophila* Wts, rat ROKα, human $p160^{ROCK}$, human PK428 [1], *Caenorhabditis elegans* LET-502 [4], *Drosophila* Genghis Khan [5], murine Citron Rho-interacting kinase (CRIK) [6], rat DM kinase-related Cdc42-binding kinase (MRCK) [7] and *C. elegans* DM kinase-related kinase (M. Shimizu and H.F. Epstein, unpublished results). The prediction based upon amino acid sequence that DMPK is a serine–threonine kinase has been verified by enzymological studies of recombinant proteins [8,9].

Several MDFPK members have been shown to interact with members of the Rho family of small GTPases. This family includes RhoA, Rac-1 and Cdc42 and belongs to the larger Ras superfamily of GTPases that share conserved amino acid sequences [10]. These MDFPK members include rat ROKα, human $p160^{ROCK}$, murine CRIK [6] and MRCK [7]. The protein kinase domain is highly conserved between CRIK and DMPK. CRIK and its close relative the Rho and Rac-binding protein Citron also share putative RhoA interaction and coiled-coil motifs with DMPK [11] (see Fig. 1).

In addition to activation by Rho-type GTPases and their associated guanylyl exchange factors (GEFs) and GTPase activating proteins (GAPs), certain serine–threonine protein kinases are also activated intracellularly by convergence or interaction with other signaling pathways. For example, ERKs and PAK show convergence of Ras–Raf kinase with Rac and Rho [12–16]. For these reasons, we have examined the direct interactions of DMPK with several signaling proteins. We find that Rac-1 and Raf-1 kinases bind to DMPK. Furthermore, coexpression with Rac-1 activates the transphosphorylation of generic protein substrates by DMPK, and phosphorylation by Raf-1 kinase stimulates the autophosphorylation of DMPK.

2. Materials and methods

2.1. Plasmid construction

The glutathione-*S*-transferase (GST)-small GTPase bacterial protein expression constructs, pGEX-Ras, pGEX-RhoA, pGEX-Cdc42 and pGEX-Rac-1, were kindly provided by Dr. J. Silvio Gutkind at NIH. The eukaryotic expression vector pCGN and the small GTPase constructs, pCGN-Rac-1, pCGN-Rac-1 G12V, pCGN-Rac-1 S17N, were obtained from Dr. Robert J. Schwartz at Baylor College of Medicine [17]. See Fig. 1 for definition of different truncated DMPK molecules. The His PKH FLAG construct (pET15b.PKH.-FLAG) was constructed and characterized in this laboratory [8]. The LPK and LPK K100A constructs were prepared as follows. The cDNA sequence corresponding to LPK (codons 1–432) was amplified by PCR from either T7.tag.LPKHT or T7.tag.LPKHT K100A which were obtained from Dr. A. Balasubramanyam at Baylor and subcloned in the *Bam*HI/*Eco*RV site of the pBSKII(SK−) plasmid (Stratagene) to yield pBSK.LPK and pBSK.LPK K100A. The *Bam*HI/*Sal*I fragment of the pBSK derivatives was inserted into the *Bam*HI/*Sal*I site of pET28a(+) (Novagen) to yield His.T7tag.LPK and His.T7-tag.LPK K100A, respectively.

2.2. Cell culture

COS-M6 cells were a gift of Dr. J. Bryan at Baylor and were maintained at 37°C in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% fetal bovine serum (Hyclone) and Antibiotic–Antimycotic drugs (Life Technologies, Inc.). COS-M6 cells

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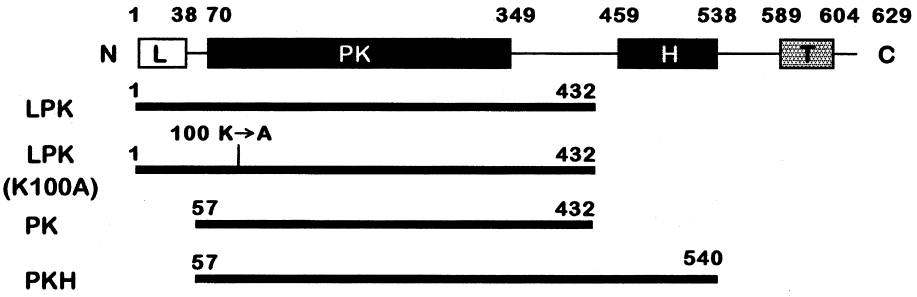

Fig. 1. Protein modules of DMPK and the constructs used in this study: L, leucine rich region; PK, serine–threonine protein kinase catalytic domain; H, helical region containing predicted coiled-coil and leucine zipper domains which also exhibit similarities to known Rho GTPase interaction sites; T, putative transmembrane anchoring region [11].

were plated at a density of 2×10^6 per 100 mm plate, and transfections were performed by calcium phosphate precipitation on the next day. Bacterial cultures containing pET15b.PKH.FLAG were performed as described [8] and bacteria carrying pET28a.His.T7tag.LPK and pET28a.His.T7tag.LPK K100A were grown at 26°C.

2.3. Protein isolation and purification

His.PKH.FLAG and the GST fusion proteins with the small GTPases were purified as described [18]. LPK and LPK K100A were purified using either His.Bind Resins (Novagen) or a HiTrap metal affinity column (Pharmacia Biotech). Ras-activated Raf-1 kinase was kindly provided by Dr. J. Avruch at the Massachusetts General Hospital. Histone H1 was from Boehringer-Mannheim.

2.4. Protein-binding assays

The GST-small GTPase fusion proteins (630 nM) were complexed with glutathione-Sepharose beads and incubated with 530 nM His.PKH.FLAG in 25 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol, 30 mM $MgCl_2$, 100 mM NaCl, 2 mg/ml bovine serum albumin and 0.5% Nonidet P-40 for 3 h. The beads were then washed (three times) in buffer without albumin and (two times) in buffer without albumin or Nonidet. The beads were suspended in 2×SDS–PAGE sample buffer [8], heated to 95°C for 5 min and the soluble proteins separated on 10% SDS–PAGE. The gel was transferred to an Immobilon-NC membrane (Millipore), reacted with a 1:1000 dilution of monoclonal anti-FLAG antibody (Kodak IBI), followed by a 1:1000 dilution of affinity-purified alkaline phosphatase-conjugated goat anti-mouse IgG (Cappel), and the alkaline phosphatase color reaction was developed according to the manufacturer (Promega).

Lysates from transfected COS-M6 cells were incubated with 540 nM His.PKH.FLAG bound to His.Bind Resins in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1 mM EDTA, 2 mM PMSF, 3 μM benzoyl-L-arginine ethyl ether, 3 μM *N-p*-tosyl-L-arginine methyl ester, 50 nM soybean trypsin inhibitor, and 2 μM each of chymostatin, leupeptin and pepstatin. The complex was incubated for 3 h at 8°C, washed with a column volume of buffer (3×), and 2×SDS–PAGE sample buffer [8] was added, the mixture heated to 95°C for 5 min and the soluble proteins were electrophoresed on 12% SDS–PAGE. Blotting was as described above. The monoclonal antibody (BabCO) to the hemagglutinin (HA) antigen was used at a dilution of 1:1000. Secondary and color reactions were as described above.

2.5. Protein kinase assays

Assays of purified proteins were performed as described and quantified by densitometry of radioautograms (Bio-Rad) [8]. Assays of proteins from COS-M6 cells were performed as follows. 48 h after transfection, cells were lysed in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM PMSF, 1% Triton X-100, 2 mM sodium orthovanadate, 2 mM sodium fluoride, 3 μM each of *N-p*-tosyl and L-arginine methyl ether, and 2 μM each of chymostatin, leupeptin and pepstatin. Cell lysate was incubated with 67 nM monoclonal anti-FLAG antibody (Kodak IBI) and 10% (v/v) of protein G-Sepharose (Pharmacia Biotech) beads at 8°C for 3 h. The bead–antibody–kinase complex was washed two times with the above buffer, layered onto a 1 M sucrose cushion, centrifuged at $13\,500\times g$ for 3 min at 8°C in a bench top centrifuge (Fisher Scientific), and washed two times with 20 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$ (kinase buffer). The beads were then incubated with 100 μM ATP, 5 μM histone H1 and 0.33 μCi/μl of [γ-^{32}P]ATP for 30 min at 30°C. An equal volume of 2×SDS–PAGE sample buffer was added and the mixture was heated to 95°C for 5 min in order to stop the reaction. The soluble proteins were electrophoresed on 10% SDS–PAGE. The gel was stained with Coomassie blue, dried, and ^{32}P incorporation into histone H1 was measured on a phosphorimager (Packard).

3. Results

3.1. Rac-1 binds to DMPK and activates the kinase activity

We found that Rac-1 was capable of binding to DMPK in vitro (Figs. 2 and 3A). As shown in Fig. 2, equal amounts of bacterially expressed GST fusion proteins RhoA, Rac-1, Cdc42 and Ras were incubated with bacterially expressed DMPK-(PKH) (defined in Fig. 1). The DMPK was shown to bind to Rac-1 but not to the RhoA, Cdc42 or Ras fusion proteins. This result was confirmed by the independent experiment using the Rac-1 which was expressed in mammalian

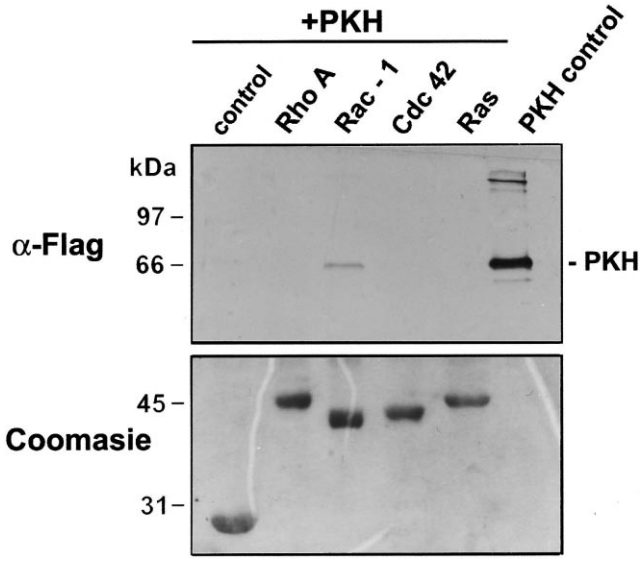

Fig. 2. Rac-1 binds DMPK. Four bacterially expressed, small GTPase protein GST fusion proteins: RhoA, Rac-1, Cdc42 and Ras at concentrations of 630 nM were each incubated with 530 nM FLAG-tagged DMPK-(PKH) and then glutathione-Sepharose beads. The presence of DMPK was detected on Western blots of the solubilized Rac-1 complexes, but not of the other GTPases by anti-FLAG (upper panel). The GTPase proteins present were detected by the Coomassie brilliant blue staining of the SDS–PAGE (lower panel). The control was GST alone.

COS-M6 cells and was shown to bind to bacterially expressed DMPK-(PKH) (Fig. 3A). These results indicate that Rac-1 can physically interact with DMPK.

The cotransfection experiments in Fig. 3B show that Rac-1

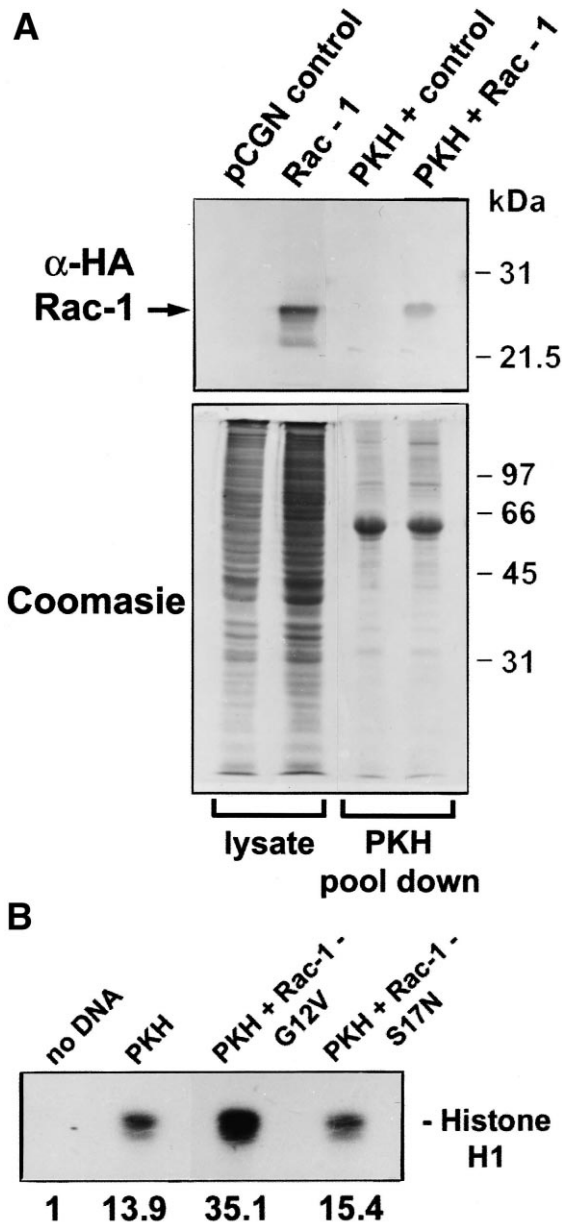

Fig. 3. (A) Rac-1 physically interacts with DMPK. Rac-1 expressed in COS-M6 cells showed detectable binding to bacterially expressed DMPK-(PKH) that was tagged with both His_6 and FLAG. The Rac-1–DMPK complexes were bound to a nickel column, solubilized and detected on Western blots by anti-HA to detect Rac-1 (upper panel). Coomassie brilliant blue-stained SDS–PAGE of the COS-M6 cell lysates (left two lanes) and recombinant DMPK (right two lanes) used in each experiment are shown for comparison (lower panel). The control was transfection by vector alone. (B) Rac-1 can activate DMPK in a GTP-sensitive manner. Constitutive mutant of Rac-1 was each cotransfected with recombinant DMPK-(PKH) in COS-M6 cells, and the resulting DMPK activity was assayed by transphosphorylation of the generic substrate histone H1. The Rac-1 G12V mutant represents the GTP-bound state, and the Rac-1 S17N mutant represents the GDP-bound state. The Rac-1 GTP state mutant gave the highest activity, whereas the Rac-1 GDP state mutant effect was similar to DMPK alone.

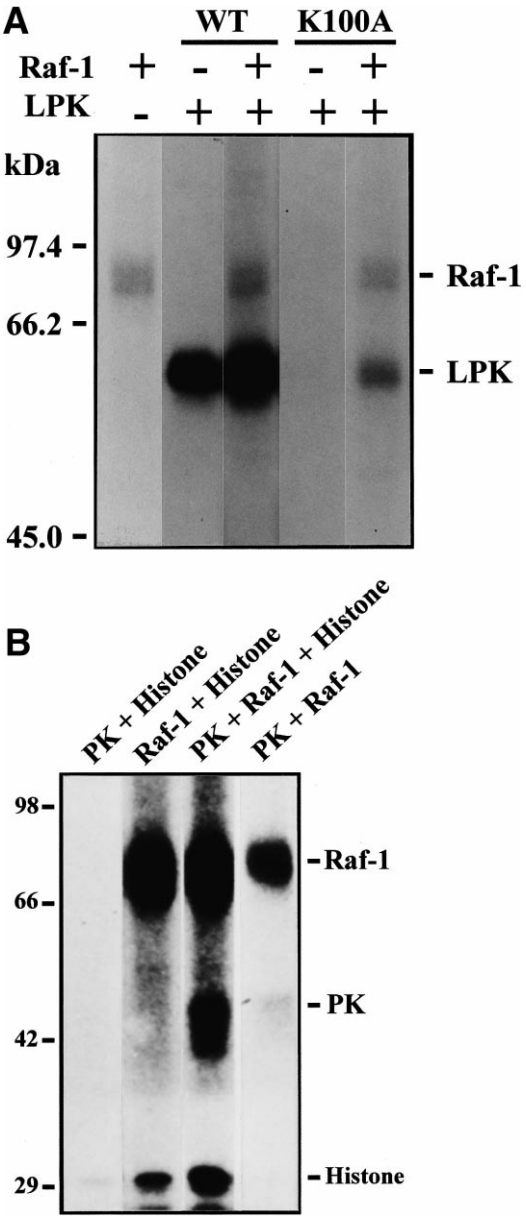

Fig. 4. A and B represent radioautograms of SDS–PAGE separations of the ^{32}P-incorporated proteins. (A) Raf-1 kinase phosphorylates DMPK. At excess DMPK to Raf, 1.5 μM of the DMPK-(LPK) K100A mutant did not show any autophosphorylation but was phosphorylated by 54 nM Raf-1. Wild-type DMPK-(LPK) also at 1.5 μM showed significant autophosphorylation that was increased by incubation with 54 nM Raf-1. The ratio of DMPK:Raf was about 28:1. (B) Raf-1 kinase and DMPK interact with each other. The effects of mixtures of 37 nM DMPK-(PK), 13.5 nM Raf-1 and 793 nM histone H1 were studied. Here, the DMPK:Raf ratio was about 3:1 but histone was in excess. Raf-1 also increased DMPK phosphorylation from non-detectable to the detected level, however, the presence of histone leads to a marked increase in the phosphorylation of DMPK and Raf-1 in addition to itself.

could lead to the activation of DMPK in a GTP-sensitive manner within cells. The individual GTPases and DMPK-(PKH) were cotransfected and the DMPK was absorbed from lysates of the cotransfected cells. Histone H1 was then added to the DMPK as a generic kinase substrate. The constitutive GTP-bound active Rac-1 mutant (G12V) showed over 2.5-fold activation of transphosphorylation of histone

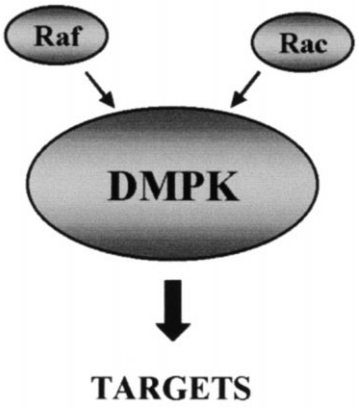

Fig. 5. Model of activation of DMPK by Rac-1 and by Raf-1 kinase, a component of the Ras-based chemical signaling pathway. We propose that this activation may regulate the interactions of DMPK with its target substrate proteins, permit cross-talk between the distinct signals and explain some of the pleiotropy of DM even within a single cell type such as type I skeletal muscle fibers [11].

H1 by DMPK-(PKH), whereas the constitutive GDP-bound inactive mutant (S17N) showed insignificant activation of DMPK. These results indicate that Rac-1 may have functional interactions with DMPK although only the Rac-1 results are consistent with guanylyl nucleotide regulation.

3.2. Raf-1 phosphorylates and activates DMPK

Fig. 4A (K100A) shows that Raf-1 kinase was able to directly phosphorylate DMPK. For this experiment, we constructed a mutant DMPK-(LPK) where K100 was replaced by A producing a kinase-deficient protein [19]. However, active Raf-1 kinase phosphorylated the mutant LPK to significant levels when both purified enzymes were incubated together. No other phosphorylatable bands could be observed in either preparation. LPK was used so that DMPK and Raf could be distinguished on SDS–PAGE.

Fig. 4A (WT) shows that wild-type DMPK-(LPK) phosphorylation was also enhanced by incubation with Raf-1 kinase and ATP. LPK phosphorylation was increased significantly by incubation with Raf to 19.45 optical density mm densitometer units when compared to either the autophosphorylation of LPK alone, 11.25 units, or the transphosphorylation of the K100A mutant by Raf, 3.2 units, or their sum, 14.45. Similar results were obtained with active DMPK-(PK) construct at lower concentrations than with LPK (Fig. 4B). Phosphorylation of both DMPK and histone H1 was highest in a ternary mixture with Raf-1 kinase in comparison to DMPK and Raf-1 (just detectable) or DMPK and histone (not detectable). In the absence of Raf-1, the levels of DMPK were insufficient for detection of their autophosphorylation whereas their transphosphorylation of histone H1 was just detectable. In the presence of Raf-1, DMPK phosphorylation was detectable. In the absence of DMPK, Raf-1 showed significant autophosphorylation and transphosphorylation of histone H1.

4. Discussion

We have shown that Rac-1 and Raf-1 kinase can physically interact with DMPK. Cotransfection with GTP-bound Rac-1 but not GDP-bound Rac-1 activated DMPK transphosphorylation of histone H1 by almost 3-fold, consistent with a potential regulatory interaction. Raf-1 kinase transphosphorylated DMPK, activated DMPK autophosphorylation and activated transphosphorylation of generic substrate protein, histone H1. The effects of Rac-1 and Raf-1 kinase with DMPK are likely to be significant because they both show binding, enzymic activation and sensitivity to known regulatory interactions.

Other members of the small GTPase family including Ras, RhoA and Cdc42 failed to show binding with DMPK. The absence of these interactions may reflect the differences in the requirements of each for GAPs and GEFs [20]. It is possible, therefore, that in the presence of additional proteins or protein complexes, these other GTPases may also be able to activate or bind DMPK.

The biochemical observations that Rac-1 and Raf-1 kinase can stimulate DMPK activity are interesting from a mechanistic viewpoint. Rac-1 together with other Rho family members such as RhoA and Cdc42 are generally considered to be associated with the actin cytoskeleton and to regulate its dynamic interactions with the plasma membrane [10,21,22]. The assembly and function of focal adhesion plaques and related structures which link organized actin-containing stress fibers, the membrane and the extracellular matrix into a complex capable of mechanical and chemical transduction are particularly dependent upon the action of Rac-1 and its relatives. Raf-1 kinase, on the other hand, has been associated with the small GTPase Ras as its activator. Ras in turn responds to receptor–tyrosine kinase complexes which are stimulated by the binding of extracellular ligands. The Ras–Raf system is generally considered to be a chemical signaling pathway linked to the MAP kinase cascade [23]. When observed within cells, this dual response has been termed 'convergence' as in the example of the activation of PAK and the subsequent transformation of rat fibroblasts by Raf kinase, RhoA and Rac-1 [16,24,25]. Other biological examples of convergence and 'cross-talk' include the stimulation by growth factors of membrane ruffling and focal adhesion stress fiber assembly [10,26] and conversely, the activation of the MAP kinase pathway by coexpression of Rho family GTPases or through cell adhesion instead of the usual growth factor–Ras–Raf route [12–15].

The biochemical findings of such dual activation of DMPK in this report suggest that this enzyme may play an important role in the cross-talk between the adhesion-dependent and chemically stimulated transduction systems (Fig. 5). Further work is required to verify these interactions in different cellular systems in order to fully assess their functional significance [13,15,16].

DMPK is likely to play an important role in the alterations of DM [27–31]. For example, in a single cell type, the type I skeletal muscle fibers, DM affects differentiation, growth and maintenance of the myofibrillar protein structures, electrical excitability of the plasma membrane and associated structures, and the metabolic response to insulin [11]. The interaction of the DMPK molecule with multiple signals provides a potential model for understanding this highly diverse pleiotropy within a single cell type.

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