Structural basis of N-Myc binding by Aurora-A and its destabilization by kinase inhibitors

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Myc family proteins promote cancer by inducing widespread changes in gene expression. Their rapid turnover by the ubiquitin–proteasome pathway is regulated through phosphorylation of Myc Box I and ubiquitination by the E3 ubiquitin ligase SCFβTrCP. However, N-Myc protein (the product of the MYCN oncogene) is stabilized in neuroblastoma by the protein kinase Aurora-A in a manner that is sensitive to certain Aurora-A–selective inhibitors. Here we identify a direct interaction between the catalytic domain of Aurora-A and a site flanking Myc Box I that also binds SCFβTrCP. We determined the crystal structure of the complex between Aurora-A and this region of N-Myc to 1.72-Å resolution. The structure indicates that the conformation of Aurora-A induced by compounds such as alisertib and CD532 is not compatible with the binding of N-Myc, explaining the activity of these compounds in neuroblastoma cells and providing a rational basis for the design of cancer therapeutics optimized for destabilization of the complex. We also propose a model for the stabilization mechanism in which Aurora-A alters how N-Myc interacts with SCFβTrCP to disfavor the generation of Lys48-linked polyubiquitin chains.

Significance

Elevated levels of N-Myc protein (the product of the MYCN oncogene) drive cancers such as neuroblastoma. Accumulation of N-Myc in these cancer cells depends upon the formation of a complex with the protein kinase Aurora-A in which the N-Myc is not properly degraded. We mapped the region of N-Myc that interacts with Aurora-A and determined the molecular structure of the complex. Because this region also interacts with cellular machinery that targets N-Myc for degradation, we sought to understand the mechanism by which N-Myc stabilizes Aurora-A. The structure explains how compounds that induce distorted conformations of Aurora-A are able to disrupt the interaction with N-Myc. This understanding may provide a basis for designing better compounds that work in this way for the treatment of neuroblastoma.

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Aurora-A in a fully active conformation that is incompatible with inhibitors of Aurora-A that disrupt the complex. Biochemical studies show an interaction between SCF<sup>F<sub>F-kink</sub></sup> and the same region of N-Myc, and we propose that the way in which Aurora-A interferes with this interaction changes N-Myc ubiquitination to promote stability.

**Results and Discussion**

**Structural Basis of the Interaction Between N-Myc and Aurora-A.** To show that Aurora-A and N-Myc interact directly, we carried out a coprecipitation experiment using a GST—Aurora-A kinase domain fusion protein incubated with a range of purified fragments from the N-Myc TAD (Fig. S1A). This method identified residues 28–89 of N-Myc as the minimal Aurora-A-interaction region (AIR), which spans MB0 through MBI but does not include MBII or beyond (Fig. L4). The binding affinity of Aurora-A for the AIR was quantified as 2.9 ± 0.5 μM by ELISA (Fig. S1B) and as 1.0 ± 0.3 μM by competition AlphaScreen assay (Fig. 1B). The presence of a Trp residue toward the C terminus of this N-Myc fragment was striking, and mutation of Trp88 to Ala abrogated the interaction in the context of the entire N-Myc TAD fragment (residues 1–137) in a precipitation experiment with GST-tagged Aurora-A catalytic domain (residues 122–403); the TAD of N-Myc did not interact with GST alone. Taken together, these results indicate that the interaction is specific (Fig. S1C).

To elucidate the structural basis of the Aurora-A/N-Myc interaction, we crystallized a complex between the Aurora-A kinase domain with surface Cys mutated to enhance stability (Aurora-A<sup>C290A/C393A</sup>) and a synthetic N-Myc peptide corresponding to residues 28–89. The crystals yielded diffraction data to 1.72-Å resolution, and we solved the structure by molecular replacement using an existing structure of Aurora-A<sup>C290A/C393A</sup> (Table S1 and Fig. S1D). Residues 28–60 of N-Myc are not observed in the structure, whereas residues 61–89 are associated with the cleft between the N- and C-lobes of the Aurora-A kinase domain formed by the αβ/αc helices, the activation loop, and the αG helix (Fig. 1C). N-Myc residues 76–89 form an α-helix, which packs onto the C-lobe of Aurora-A at a surface formed by Tyr334, Glu335, and Tyr338 with its N terminus pointing toward the substrate-binding region. Both ends of the α-helix are capped by Trp residues (Trp77 and Trp88), whose side chains pack against the surface of Aurora-A. Key intermolecular interactions include a salt bridge between Glu73 of N-Myc and Lys143 from the Gly-rich loop on the N-lobe of Aurora-A, an additional interaction between Gln335 of Aurora-A and Trp88 of N-Myc, and the insertion of the Trp77 side chain of N-Myc into the hydrophobic P±1 pocket in the activation loop region of Aurora-A (Fig. 1D). A pair of prolines (Pro74 and Pro75) breaks the α-helix at its N terminus and directs the chain toward the Aurora-A N-lobe. Residues 69–71 are hydrogen-bonded into a turn, and residues 61–67 are bound into a groove between the N-lobe and the surface formed by the activation loop.

The region of N-Myc observed in the crystal structure starts at the C terminus of MBI and is not conserved in c-Myc (Fig. S2A). Our initial mapping suggested that this region was insufficient for binding, so we used a more sensitive assay based on changes in fluorescence polarization (FP) using synthetic peptides of N-Myc to confirm that residues 61–89 of N-Myc bind Aurora-A independently, with a measured K<sub>d</sub> of 12 μM (Fig. S2B). N-Myc peptides with E73K or W77A mutations abrogated binding to Aurora-A, consistent with their observed contributions to the interface in the crystal structure (Fig. S2B). Similarly, the contributions of Aurora-A Y334 and Q335 to the interaction were confirmed.

The section of the AIR that was not resolved in the crystal structure, residues 28–60 of N-Myc, includes the MB0 and MBI regions and is conserved in c-Myc (Fig. S2A). Having shown that the C-terminal part of the AIR was able to bind Aurora-A independently, we looked for other subfragments that might contribute to binding with a sensitive pull-down assay using peptides spanning the region, under less stringent conditions than the first set of pull-downs used for mapping the interaction (Fig. S2C). In addition to residues 61–89, a region corresponding to MB0 was also capable of independent interaction with Aurora-A. Using the FP assay, we confirmed that a peptide spanning residues 19–47 of N-Myc, including MB0, binds independently to Aurora-A (Fig. S2D). The presence of conserved aromatic residues in this region is striking (F82, Y29, F35, Y36), and these residues were shown to contribute to the interaction with Aurora-A (Fig. S2D). In contrast to the established roles of the regions flanking it, MBII itself does not appear to contribute to the interaction with Aurora-A, because a peptide corresponding to residues 44–64 showed no binding in the peptide coprecipitation assay (Fig. S2C). Consistent with this result, neither the phosphorylation status of the phosphodegron residues Thr58 and Ser62 nor mutation of residues 52–56 to Ala affected Aurora-A binding of...
the N-Myc 28–89 peptide (Fig. S2E). We postulate that the flanking regions on either side of MBI form specific interactions with Aurora-A, linked by the MBI region itself, which interacts in a phosphorylation-dependent manner with FbxW7 but makes no critical interactions with Aurora-A.

**N-Myc Activates Aurora-A and Competes with TPX2, the Targeting Protein for Xklp2.** When bound to N-Myc, Aurora-A is in a fully active conformation similar to that observed for the Aurora-A/TPX2 complex (Fig. 24) (23). The microtubule-associated protein-targeting protein for Xklp2 (TPX2) binds to Aurora-A through its first 43 residues: amino acids 7–21 at a site on the N-lobe and amino acids 30–43 at a site between the two lobes. The conformation adopted by Aurora-A when bound to TPX2 is incompatible with the interaction of N-Myc residues 61–67 (Fig. 2B). There is a clear steric clash, because Leu61 of N-Myc binds to the same pocket on the Aurora-A surface as Trp34/Phe35 of TPX2. To accommodate the marked increase in size in going from the single Leu side chain of N-Myc to the two bulky side chains of TPX2, the side chain of Aurora-A residue His187 rotates out of the pocket in the TPX2-bound conformation. Side chains on the αC-helix of Aurora-A such as His176 and Arg179 are also observed in different positions, suggesting that the N-terminal stretch of TPX2 (Tyr8/Tyr10) that binds on one side of the αC-helix is incompatible with binding of N-Myc to the other side of the αC-helix. Consistent with this structural analysis, the first 43 amino acids of TPX2 and the AIR of N-Myc compete for binding to Aurora-A (Fig. 2C), and they also have comparable affinities, because $K_a$ values of 2–3 μM were measured for both proteins in ELISAs using immobilized Aurora-A (Fig. S1B). Competition with TPX2 was observed for the individual regions of the AIR both N- and C-terminal to MBI (Fig. 2C), suggesting that the binding site for MB0 on Aurora-A also overlaps with that of TPX2. The AIR of N-Myc, like TPX2, initially activates unphosphorylated Aurora-A, so there is also functional overlap between these two Aurora-A–binding proteins (Fig. 2D and Fig. S3). The crystal structure reported here does not reveal the mechanism by which N-Myc activates Aurora-A, because residues 61–89 of N-Myc were not sufficient to activate Aurora-A, and we used Aurora-A prephosphorylated on Thr288 to form the complex. However, it is clear that N-Myc, like TPX2, is able to trigger Aurora-A activation through protein–protein interactions, and the result is a stabilized conformation of Aurora-A in which the activation loop forms a platform for the binding of N-Myc residues 61–89 (Fig. 2E). Furthermore, as shown in Fig. S3, Aurora-A is able to phosphorylate residues within the 28–89 region of N-Myc efficiently in vitro.

The Conformation of Aurora-A Bound to N-Myc Is Incompatible with Inhibitors That Destabilize the Interaction. Previous studies showed that ATP-competitive inhibitors of Aurora-A such as MLN8054, MLN8237, and CD532 disrupt the formation of the Aurora-A/N-Myc complex, resulting in destabilization of N-Myc in cell models. Crystal structures of Aurora-A in complex with MLN8054

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**Fig. 2.** Structural and functional comparison of Aurora-A interactions with N-Myc and TPX2. (A) Superposition of crystal structures of Aurora-A bound to N-Myc (colored red) and TPX2 (colored pink). Aurora-A bound to TPX2 is colored a lighter shade of green. (B) Magnified view of the superposed structures. The side-chain motions accompanying the transition from the N-Myc to the TPX2 complex are indicated by black arrows. (C) Coprecipitation experiments to investigate competition between TPX2 (residues 1–43) and N-Myc fragments for binding to Aurora-A (residues 122–403), analyzed by SDS/PAGE. The gel shows proteins coprecipitated with biotinyl-N-Myc peptides (as indicated) immobilized on streptavidin beads following incubation with 12-μM Aurora-A catalytic domain in the presence or absence of a fivefold molar excess of TPX2 1–43 protein. (D) Kinase assays to measure the activity of the 0.625-μM initially unphosphorylated Aurora-A catalytic domain in the presence of [γ-32P]ATP and varying concentrations N-Myc peptides (residues 28–89, 19–47, or 61–89) or TPX2 (residues 1–43), reported by scintillation counting. Data are the mean of two experiments ± SE. (E) Schematic illustration of the activating interactions of N-Myc and TPX2 with Aurora-A. The unphosphorylated activation loop of Aurora-A is flexible (dashed black line) but becomes ordered upon kinase activation through the binding of protein partners and autophosphorylation.
or CD532 show distorted conformations of the kinase, notably in the positions of the activation loop and the Gly-rich loop (20, 21). In contrast, most Aurora-A inhibitors, such as CCT137690, do not substantially affect the structure of the protein (24). There is no available crystal structure of MLN8257 bound to Aurora-A, but the chemical structures of MLN8054 and MLN8237 are almost identical, and it is likely that MLN8237 induces a similar conformation in Aurora-A. We compared the structures of Aurora-A in complex with N-Myc, MLN8054, CD532, and CCT137690 (Fig. 3A). Aurora-A grips N-Myc through interactions involving both the N- and C-lobes of the kinase (colored orange in Fig. 3A). Binding of CCT137690 does not affect the relative orientation of the two kinase lobes. The activation loop of Aurora-A is disordered in the Aurora-A/CCT137690 structure because it is neither phosphorylated nor stabilized by a protein such as TPX2 or N-Myc, and there is no obvious mechanism by which CCT137690 could affect the activation loop. However, when Aurora-A is bound to MLN8054 or CD532, the surfaces that form the binding site for residues 61–89 of N-Myc are moved apart through motions that twist the two kinase lobes relative to one another and displace the activation loop (Fig. 3B). The rearrangement of this binding site provides a possible mechanism by which compounds such as MLN8054 disrupt the Aurora-A/N-Myc complex by acting as a wedge that forces the kinase into an inactive conformation. The key differences between CCT137690 and MLN8054 are that only the latter makes a specific contact with the displaced activation loop; although both compounds contact both the hinge and the Gly-rich loop, the wedge effect is a function of the 3D shape of MLN8054 in contrast to the flatter scaffold of CCT137690.

Consistent with the structural analysis, MLN8054 but not CCT137690 disrupted the direct interaction of the catalytic domain of Aurora-A with its binding sites within the AIR (Fig. 3C). Indeed, Aurora-A bound to MLN8054 displayed two- to threefold reduced affinity for both of the binding sites within the AIR of N-Myc compared with Aurora-A alone (Fig. S4). Next, we used proximity ligation assays to quantify complex formation between endogenous N-Myc and Aurora-A proteins in a neuroblastoma cell line (Fig. 3 D and E) and observed significantly fewer interactions in cells treated with MLN8054 or MLN8237 than in untreated cells, whereas the number of interactions was unaffected by treatment with CCT137690.

A Model for the Regulation of N-Myc Ubiquitination by Aurora-A. In this study, we showed that Aurora-A binds N-Myc irrespective of its phosphorylation state and interacts with regions of N-Myc that flank MBI. The binding interaction between the Aurora-A catalytic domain and N-Myc residues 61–89 that we have resolved is immediately adjacent to the phospho-degron motif centered on Thr58. Phosphorylation of this motif is required for recognition of N-Myc by SCF^{FbxW7}. Thus, binding of Aurora-A might affect the interaction of N-Myc with SCF^{FbxW7}. Using purified proteins, we discovered that SCF^{FbxW7}, like Aurora-A, interacts with N-Myc irrespective of the phosphorylation state of Thr58/Ser62 (Fig. 4A). Binding of SCF^{FbxW7} to N-Myc that is phosphorylated on Thr58/Ser62 was not affected by Aurora-A, as is consistent with nonoverlapping binding sites on N-Myc. In contrast, Aurora-A competed with SCF^{FbxW7} for binding to unphosphorylated N-Myc. This observation suggested that the interaction of SCF^{FbxW7} with unphosphorylated N-Myc might depend on a binding site that overlaps with one or both of the Aurora-A binding sites that flank MBI. Binding of SCF^{FbxW7} was mapped to residues 61–89 of N-Myc; binding to residues 48–89 of N-Myc was also observed, but an unphosphorylated MBI peptide (residues 44–64) did not bind (Fig. 4B). Aurora-A efficiently competed with SCF^{FbxW7} for binding to residues 48–89 of N-Myc in a dose-dependent manner, whereas a control protein bovine serum albumin (BSA) did not compete (Fig. 4C). Taken together, these results show that there is competition between Aurora-A and SCF^{FbxW7} for binding to residues 61–89 of N-Myc, the region bound to Aurora-A in the crystal structure.

These observations suggest a working model by which Aurora-A could stabilize N-Myc by altering its interaction with SCF^{FbxW7}. We put forward this model as a basis for further investigation.
Aurora-A alters the interaction of N-Myc with SCF\textsuperscript{FbxW7}. (A, Upper) Competition co-precipitation experiments using biotinylated N-Myc AIR peptides immobilized on streptavidin beads and incubated with 2 μg of the SCF complex incorporating GST-tagged FbxW7 in the presence or absence of 12 μM untagged Aurora-A catalytic domain. Equal loading of resin was assessed by Coomassie Blue staining. (Lower) Binding of SCF\textsuperscript{FbxW7} to N-Myc was visualized by Western blot (WB) using anti-GST antibody. (B, Upper) Co-precipitation experiments using biotinylated N-Myc AIR fragment peptides immobilized on streptavidin beads and incubated with 2 μg SCF complex incorporating GST-tagged FbxW7. Equal loading of resin was assessed by Coomassie Blue staining. (Lower) Binding of SCF\textsuperscript{FbxW7} to N-Myc peptides was visualized by Western blot using an anti-GST antibody. Note that in A and B, despite the uneven appearance of the peptides in the upper panels because of differences in migration and sensitivity to Coomassie Blue staining, equal amounts were loaded into the experiments. (C) Coprecipitation experiments showing competition between SCF\textsuperscript{FbxW7} and Aurora-A for binding to N-Myc 48–89. Biotinylated N-Myc 48–89 peptide was immobilized on streptavidin beads and incubated with 2 μg of the SCF\textsuperscript{FbxW7} complex in the presence of Aurora-A at a range of concentrations or with BSA. FbxW7 coprecipitating with the peptide was visualized by anti-GST Western blot. (D) A working model to explain how the interaction between Aurora-A and N-Myc residues 61–89 may be modified effectively with K48-linked polyubiquitin chains, and competitive binding of Aurora-A to this region interferes with K48-linked polyubiquitination of N-Myc, leading to reduced proteasomal degradation.
For in vitro peptide coprecipitation assays, 40 μM Biotinyl Avi-tagged Aurora-A 122–403 was used as described previously (35). Appropriate concentrations of bio-

Competition AlphaScreen Assay. Biotinyl Avi-tagged Aurora-A 122–403 and 3xFlag-N-Myc 28–89 for use in competition ex-

Kinase Assay. [32P]ATP kinase assays were performed as described (36) with modifications as follows. Measurement of Aurora-A activity was carried out using reactions containing 0.625 μM unphosphorylated Aurora-A catalytic domain (61 M-Myelin basic protein (Sigma)), and 0.3 μM Biotinyl Aurora-A 122–403 were mixed together and incubated at RT for 1 h in white, opaque 96-well half-area plates. Streptavidin donor beads (20 μg/mL) and anti-Flag acceptor beads (PerkinElmer) were added simultaneously to each reaction under subdued lighting conditions, and the plate was incubated for a further 90 min. The signal from each reaction was read using an EnVision multilabel plate-reader (PerkinElmer). Data points represent the mean of three experiments; error bars indicate SD. Data were analyzed using GraphPad Prism 6, and the dependence of the AlphaScreen assay on concentration of competing Aurora-A 122–403 was fitted by nonlinear regression to a one-site ligand equation in which, under the se-

In Situ Proximity Ligation Assay. Kell cells were cultured in RPMI 1640 supplemented with heat-inactivated FBS. Compounds (as indicated) at 500 nM were added 4 h before fixation. Cells were fixed with 4% para-

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