

IAPs as E3 ligases of Rac1

Shaping the move

Tripat Kaur Oberoi-Khanuja and Krishnaraj Rajalingam*

Emmy Noether Group of the DFG; Institute of Biochemistry II; Goethe University Medical School; Frankfurt, Germany

Inhibitors of Apoptosis Proteins (IAPs) are well-studied E3 ubiquitin ligases predominantly known for regulation of apoptosis. We uncovered that IAPs can function as a direct E3 ubiquitin ligase of RhoGTPase Rac1. cIAP1 and XIAP directly conjugate polyubiquitin chains to Lysine 147 of activated Rac1 and target it for proteasomal degradation. Consistently, loss of these IAPs by various strategies led to stabilization of Rac1 and mesenchymal mode of migration in tumor cells. IAPs also regulate Rac1 degradation upon RhoGDI1 depletion and CNF1 toxin treatment. Our observations revealed an evolutionarily conserved role of IAPs in regulating Rac1 stability shedding light on to the mechanisms behind ubiquitination-dependent inactivation of Rac1 signaling.

Inhibitors of Apoptosis

Inhibitors of Apoptosis Proteins (IAPs) are a class of evolutionarily conserved, multi-functional proteins primarily characterized by the presence of a Baculoviral IAP repeat (BIR) domain.^{1,2} BIR domains are classical protein-protein interaction motifs with which IAPs directly bind to various proteins including caspases. Five IAPs including XIAP, cIAP1, cIAP2, ILP2 and ML-IAP possess a RING domain with E3 ubiquitin ligase activity.³ Upon apoptosis induction, natural IAP antagonists Smac/DIABLO, Omi/HtrA2 and ARTS translocate from the mitochondria to release IAP-mediated inhibition of caspases.^{4,5} XIAP is considered as a direct inhibitor of caspases and recent studies revealed an *in vivo* role for the RING domain of XIAP in

regulating caspase activation.^{6,7} cIAPs are highly implied in regulating the activation of both canonical and non-canonical pathway of NFκB by functioning as the direct E3 ubiquitin ligases of RIPK1 and NIK respectively.⁸ IAPs also function as the E3 ubiquitin ligases of RIP2 to regulate innate immunity signaling.⁹ Further, IAPs are highly expressed in tumor cells and cIAP1 was recognized as an oncogene.^{1,10} Recent studies revealed that depletion of IAPs has led to sensitization of tumor cells to conventional chemotherapeutic drugs. These results have emboldened scientists to pursue for IAP antagonists to treat cancers and several of these drugs (anti-sense nucleotides and Smac mimetics) are already in clinical trials.²

IAPs Directly Regulate Rac1 and Cell Shape

We have recently uncovered that IAPs also control the RAF/MEK/ERK1/2 pathway and cell migration by directly regulating the stability of C-RAF kinase.¹¹ These observations have raised an important question. Does treatment with IAP antagonists also lead to an increase in C-RAF levels and cell migration? To address this issue we have treated several tumor cell lines with various concentrations of IAP antagonist compound (BV6/IAC). Previous studies have shown that these compounds trigger the autoubiquitination of cIAPs leading to NIK stabilization and activation of the non-canonical NFκB pathway. This in turn leads to the production of TNFα and cell death in a cell type dependent manner.^{12,13} Treatment of HeLa cells with IACs promoted cell death

Keywords: IAPs, XIAP, cIAP1, Rac1, RhoA, RhoGTPases, ubiquitination, proteasome, E3 ligase, ubiquitin, cell migration, plasticity, amoeboid, mesenchymal

Submitted: 01/08/12

Revised: 03/06/12

Accepted: 03/11/12

<http://dx.doi.org/10.4161/sgtp.19988>

*Correspondence to: Krishnaraj Rajalingam;
Email: krishna@biochem2.de

Comment on: Oberoi TK, Dogan T, Hocking JC, Scholz RP, Mooz J, Anderson CL, et al. IAPs regulate the plasticity of cell migration by directly targeting Rac1 for degradation. *EMBO J* 2012; 31:14–28; PMID:22117219; <http://dx.doi.org/10.1038/emboj.2011.423>.

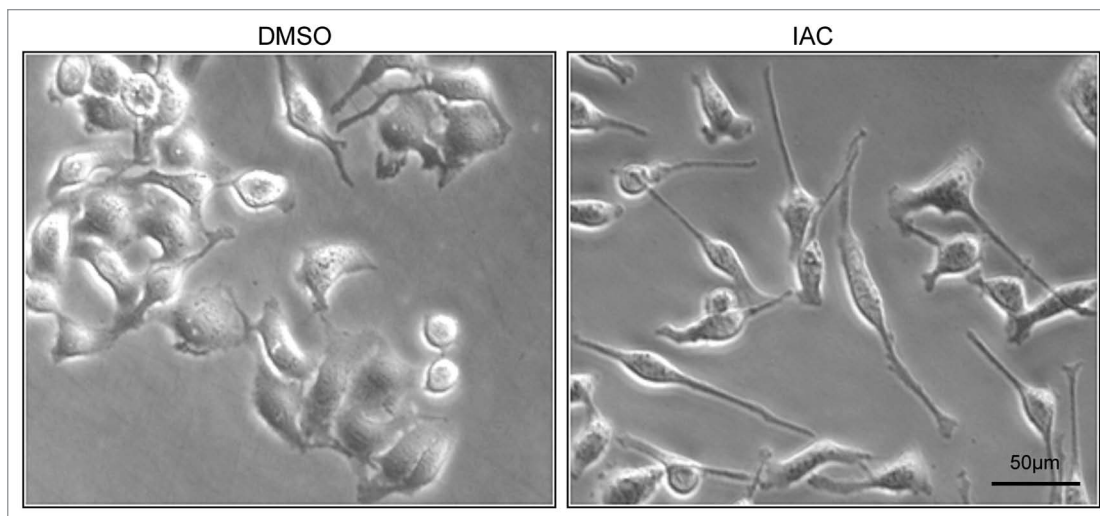


Figure 1. Loss of IAPs trigger AMT in HeLa cells. HeLa cells seeded on gelatine coated plates were treated with 5 μ M of IAC. The morphological changes were monitored by phase contrast microscopy.

in a TNF α -dependent manner and when combined with a pro-death ligand like TRAIL there was massive sensitization of these cells to apoptosis. However, when employed at sublethal doses, IACs triggered a complete loss of cIAP1 and partial loss of other IAPs (cIAP2 and XIAP) in a concentration and time dependent manner leading to an increase in C-RAF levels and enhanced transwell migration of HeLa cells.¹⁴ Intriguingly, most of the IAC treated HeLa cells exhibited highly elongated morphology with protruding lamellipodia irrespective of the substrate on which they were grown (Fig. 1). These effects are also detected in other tumor cell lines (HT1080, WM852 and A549) as well as in immortalized human mammary epithelial cells. While the migration of IAC-treated HeLa cells is dependent on MAPK activation, the formation of elongated morphology was not inhibited by the treatment of cells with MEK inhibitor U0126. Tumour cells exhibit primarily two different modes of migration depending on the extracellular cues: RhoA dependent amoeboid mode and Rac1 dependent mesenchymal mode and the cells switch from one mode to another depending on matrix density and composition.^{15,16} As IAC treated cells exhibited amoeboid to mesenchymal transition (AMT), we have tested for the activation status of Rac1 and RhoA. As expected, Rac1-GTP levels increased upon IAC treatment with concomitant reduction in RhoA-GTP

levels. However, to our surprise, there was a modest increase (1.5- to 2-fold) in the total Rac1 protein levels in IAC treated cells which has led to the notion that IAPs might regulate the stability of Rac1 directly. Indeed, loss of XIAP or cIAP1 by siRNAs or genetic deletion has led to an increase in total Rac1 levels in several cell lines.¹⁴ Further studies revealed that IAPs could directly bind Rac1 in a nucleotide independent manner and catalyze the conjugation of polyubiquitin chains leading to its proteasomal degradation. By applying mass spectrometric approaches, we have detected that XIAP and cIAP1 can directly conjugate ubiquitin to Lysine 147 of Rac1, a major ubiquitination site already shown to be responsible for regulating Rac1 stability.^{14,17}

Ubiquitin Dependent Regulation of Rac1: Role for IAPs and HACE1

Rac1 is a well-studied RhoGTPase, which controls numerous cellular functions.¹⁸ Apart from nucleotide binding and isoprenylation, Rho GTPases are also regulated by phosphorylation, SUMOylation and ubiquitination.¹⁹⁻²¹ The binding of nucleotides to Rac1 is regulated by several GEFs and GAPs and a recent study from Angeliki Malliri's group has revealed a role for PIAS3 for SUMOylation of Rac1.²⁰ Though Rac1 has been shown to be polyubiquitinated, the E3 ubiquitin ligases responsible for this process

remained elusive. Ubiquitination is a most versatile form of posttranslational modification where proteins are covalently conjugated to ubiquitin in a three-step process.²² Proteins can be conjugated with single ubiquitin on one lysine or on multiple lysines (mono- or multi-mono ubiquitination) or as polyubiquitin chains of various kinds.²³ Proteins conjugated with K-48 or K-29 linked ubiquitin chains are normally doomed for proteasomal degradation while the proteins tagged with K-63 linked or M1 linked chains lead to assemblage of protein complexes and signal transduction.²⁴ E3 ubiquitin ligases constitute the third step of the ubiquitination process and play an obligatory role in catalyzing the conjugation of ubiquitin to a lysine residue in the target proteins. The chain initiation, elongation and topology are, however, primarily controlled by the E2 enzymes.²⁵ RhoGTPases were initially observed to be degraded via proteasomes during host-pathogen interactions. Cytotoxic Necrotizing factor 1 (CNF1), a toxin secreted by uropathogenic *E. coli*, has been shown to constitutively activate RhoGTPases by deamidation leading to their proteasomal degradation.²⁶ While Smurf1 and Cullins (Cul3) were shown to regulate the polyubiquitination and degradation of RhoA, the E3 ligases driving Rac1 polyubiquitination were not known.²¹ Our observations unexpectedly revealed a role of IAPs in the inactivation of Rac1 by direct polyubiquitination and

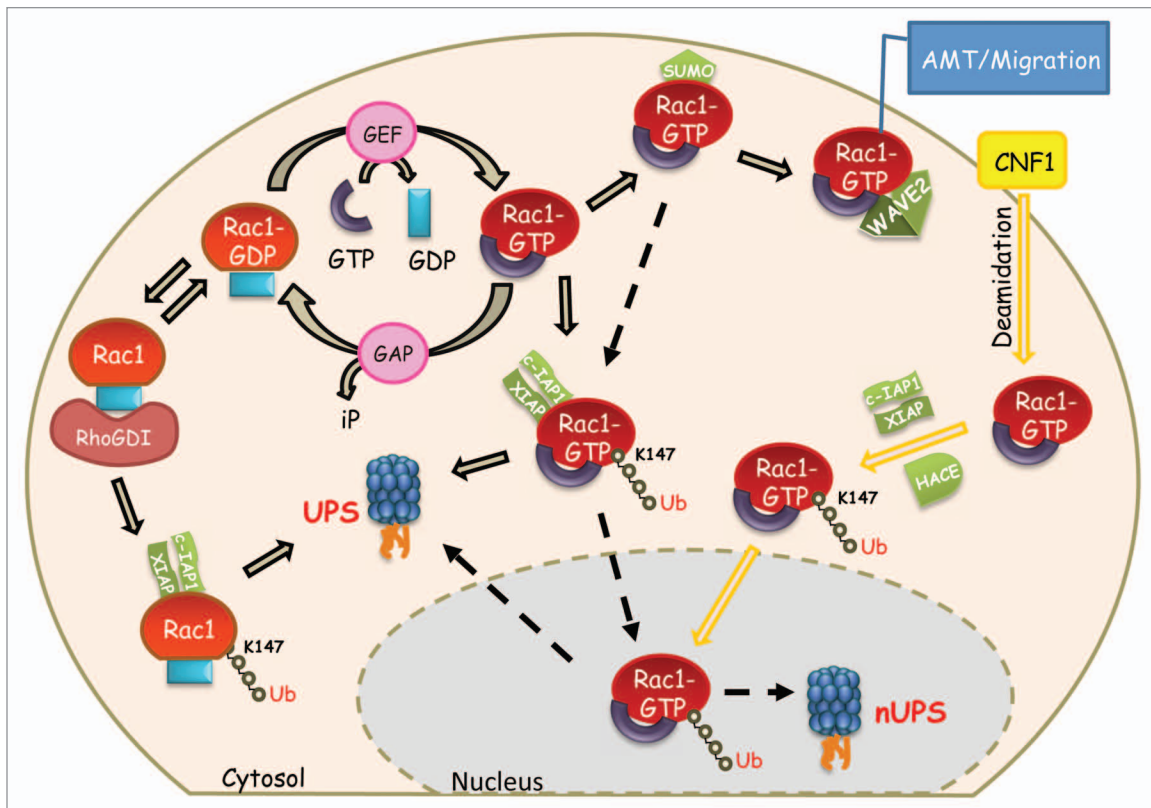


Figure 2. IAPs and HACE1 directly interact with Rac1 and promotes its polyubiquitination. Binding of Rac1 to GEFs and GAPs controls activation-inactivation cycle of Rac1. Most of Rac1 is sequestered to RhoGDI in the cytosol in GDP bound form preventing it from degradation. Activated Rac1 is membrane bound and is maintained in the GTP-bound state by PIAS-mediated SUMOylation leading to tumor cell migration and invasion. Free Rac1 in the cytosol is unstable and is ubiquitinated by IAPs leading to its degradation via the ubiquitin-proteasome system (UPS) in the cytosol or nucleus. CNF1 toxin leads to deamidation of Rac1 at Q61, which leads to persistent activation, nuclear shuttling and thus predisposing it for degradation by ubiquitination via IAPs or HACE1. IAPs directly ubiquitinate Rac1 at lysine 147 leading to its proteasomal degradation. For simplicity isoprenylation of Rac1 is avoided.

proteasomal degradation under various conditions including CNF1 toxin treatment.¹⁴ Apart from IAPs, elegant work from Emmanuel Lemichez and colleagues has also revealed a role for HACE1 for polyubiquitination of Rac1.²⁷ HACE1, unlike IAPs carries a HECT domain to catalyze polyubiquitination of Rac1. Interestingly, HACE1 preferentially binds to the GTP-bound form of Rac1 while IAPs can bind to Rac1 irrespective of its activation status. These results suggest that IAPs are probably binding to the C-terminus of Rac1. Further IAPs can also bind to Rac2 and Rac3 though these isoforms are not regulated by IAP expression. In addition, IAP overexpression has failed to influence the protein levels of Rac1b, a tumor associated splice variant of Rac1. Binding of XIAP to Rac1 was independent of BIR3, UBA and RING domains and further studies are required

to clarify if any of the three BIR domains exhibit preferential specificity for binding to Rac1. Though IAPs bind to Rac1Q61L and Rac1T17N, activated Rac1 seems to be a better substrate of cIAP1 at least in vitro in the presence of Ubc5Ha as an E2 enzyme. As XIAP can also ubiquitinate directly, it is plausible that XIAP-cIAP1 complex would function as a more efficient ubiquitin ligase in vivo and heteromerization between XIAP and cIAP1 has been demonstrated before.^{28,29} Depletion of either XIAP or cIAP1 or both has led to an identical increase in Rac1 levels. These results suggest that IAPs are functionally non-redundant in regulating Rac1 levels though we cannot completely rule out the possibility that these two IAPs might control two different subcellular pools of Rac1. Most of the Rac1 protein is localized to the cytosol in complex with RhoGDI and depletion of RhoGDI1 leads to a strong

degradation of RhoGTPases including Rac1.³⁰ Our observations suggest that XIAP is required for degradation of Rac1 upon RhoGDI1 depletion. As IAPs are predominantly cytosolic, it is tempting to propose that XIAP might primarily target the cytosolic pool of Rac1 in cells (Fig. 2). Obviously there will be a significant contribution of other Rac1 effectors like caveolin in regulating the interaction between Rac1 and its E3 ligases (Fig. 2).³¹ In these lines, it is interesting to point out that an interaction between XIAP and caveolin-1 has been demonstrated before.³² Previous studies have revealed that nuclear localization and interaction with karyopherin $\alpha 2$ are prerequisites for CNF1-mediated Rac1 degradation.³³ It is not clear, if HACE1 is localized to the nucleus and if HACE1 interacts with other known Rac1 effectors. In these lines, it is interesting to point out that cIAP1 has been shown to be shuttling

to the nucleus in many cell types. IAPs have been shown to synthesize various kinds of ubiquitin chains in vitro and in vivo. While HACE1 exploits UbcH7 as an E2 enzyme, dimerized RING domains of IAPs primarily interact with Ubc5H subfamily members for its auto-, cross- and substrate ubiquitination. Improved mass spectrometric analysis would enable to unveil the kind of ubiquitin chains being synthesized on Rac1 at endogenous levels by these E3 ligases. In addition, further analysis of in vitro ubiquitination of Rac1 with IAPs revealed that ubiquitin could also be conjugated by IAPs to other lysines in Rac1. Currently, we are investigating the biological significance of these observations. As Rac1 is also SUMOylated by PIAS (primarily at Lysines 188, 183 and 184 or 186 in the Poly basic region) it would be interesting to decipher the cross-talk between ubiquitination and SUMOylation in regulating Rac1 function. SUMOylated Rac1 may be protected from being recognised by the E3 ubiquitin ligases so as to sustain Rac1-GTP levels to facilitate cell migration and invasion.³⁸

Role of XIAP in Regulating Cell Migration during Vertebrate Development

Mammals express eight IAPs and mice deficient in individual IAPs lack any major developmental phenotypes. As IAPs directly regulate Rac1 levels, lack of any major migration phenotypes during development of mice is indeed intriguing. This could be attributed to the presence of other E3 ligases for Rac1 (like HACE1) and cross regulation between the IAPs. IAPs are constitutively organized into heteromeric protein complexes (IAPosomes) and loss of one IAP leads to an increase in the protein levels of another IAP.²⁹ For instance, loss of cIAP1 led to an increase in the protein levels of cIAP2 and vice versa.³⁴ Our unpublished observations further suggest that XIAP can also regulate the protein levels of cIAP1. This is evident in the experiments performed with MEFs derived from XIAP deficient mice as increased Rac1 levels observed in these early passage MEFs were cleared off at later passages possibly due to upregulation of cIAP1. Thus we would expect

severe developmental defects in XIAP-cIAP1 double deficient mice. Further, it would be interesting to learn if there is any cross talk between IAPs and HACE1 and their relative contribution in regulating Rac1 levels under various conditions (Fig. 2). IAP mediated regulation of Rac1 is evolutionarily conserved as DIAP1, an IAP from *Drosophila* has been shown to bind to Rac1 in a nucleotide independent manner.³⁵ We have extended on these observations and uncovered that *Danio rerio* XIAP can also bind to Rac1 in a nucleotide independent manner. Further, in collaboration with Reinhard Koster's lab we have examined the role of Dr-XIAP in regulating cerebellar granule cell migration during zebrafish brain development. During vertebrate development, the cerebellar granule cells (CGNs) exhibit highly directional migration from the Upper Rhombic Lip to form the cerebellum.³⁶ These cells primarily express Rac1 isoform and migrate collectively as chain-like structures in a Cadherin-2 dependent manner.³⁷ Interestingly, increasing XIAP in these cells has led to the formation of rounded cells, which delaminate from the tissue architecture and are often found in the cerebrospinal fluid present in the fourth ventricle. These effects are clearly Rac1 dependent as co-expression of Rac1 with XIAP can partially rescue the phenotype.¹⁴ Further, depletion of XIAP using siRNAs has led to a strong increase in Rac1 levels in the immortalized cerebellar granule cells derived from mice. The delamination phenotype observed in XIAP overexpressing CGNs resembles medulloblastoma which originates from CGN cells. These observations reveal an in vivo role of XIAP in regulating progenitor cell migration by directly controlling Rac1 homeostasis during brain development of a vertebrate organism.

Role of IAPs in Regulating Tumor Cell Migration and Metastases

The role of IAPs in regulating cell migration remains controversial, as two other labs have detected a role for IAPs in promoting cell migration.^{39,40} The apparent discrepancy can be attributed to the difference in the cell types employed or on the strategy adopted to downregulate

IAPs. While our studies are primarily based on HeLa cells, Lopez et al. employed MCF-7 cells in their analysis. Similarly, Liu et al. primarily employed shRNAs or genetic deletion in human tumor cell lines to downregulate XIAP in contrast to our loss of function studies, which are primarily based on transient downregulation of IAPs by employing siRNAs and IAC. However, this issue needs to be reconciled as we have detected an increase in Rac1 levels upon IAP depletion in multiple cell types including MCF-7 cells and SV40 transformed MEFs derived from XIAP deficient mice have also exhibited enhanced migration and wound healing under our experimental settings. Further, by creating a transgenic adenocarcinoma of the mouse prostate (TRAMP) mice that lack XIAP, Collin Duckett and colleagues have shown that loss of XIAP has actually led to an aggressive form of disease.⁴¹ In a more recent study, Dario Altieri and colleagues have shown that XIAP-survivin complex can promote metastases in a NFκB dependent manner.⁴² Whether our current results have any relevance to these observed phenotypes in mice deserves further investigations. As downregulation of IAPs enhanced cell migration, one obvious concern is that treatment with IAP antagonists might unexpectedly promote the metastases of surviving tumor cells. However, this may not necessarily be the case as increased Rac1 levels can be compensated by co-regulation of GAPs and GEFs. Further, Rac1 activity has been shown to both promote and inhibit tumor cell invasion in a cell type dependent manner. For instance, ectopic expression of Tiam1, a Rac1 GEF or activated Rac1 actually led to an increase in E-cadherin mediated cell adhesion and loss of invasiveness in RAS-transformed MDCK cells.⁴³ However, Rac isoforms are found to be highly expressed in tumor cells and overexpression of Rac1 GEFs like P-Rex is often detected in invasive breast cancer cells.^{38,44} Rac1 is also shown to be required for K-Ras-mediated lung tumor formation.⁴⁵ Thus, IAP-mediated Rac1 regulation might regulate tumor cell migration in a tissue/cell type dependent manner. As IAP antagonists are primarily pursued in combination with other chemotherapeutics in clinical trials, one

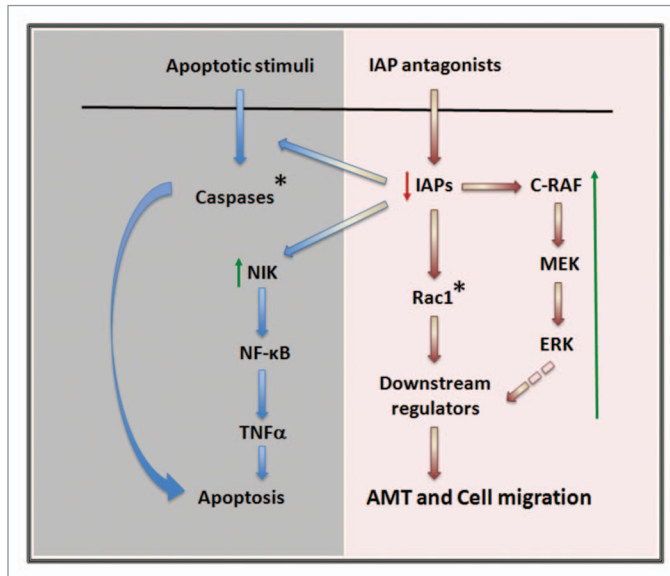


Figure 3. IAP antagonists induce cIAP1 degradation and NF κ B activation leading to TNF α -mediated cell death. On the other hand, loss of cIAP1 also stabilizes Rac1 and C-RAF leading to mesenchymal morphology and enhanced cell migration. In the absence of cell death, IAP antagonists might trigger mesenchymal mode of migration which might lead to promotion/inhibition of tumor metastases depending on the cell/tissue background. *denotes activation/upregulation of the proteins.

would expect sensitization of tumor cells to apoptosis. However, if the cells survive there may be a risk of promoting tumor cell invasion by loss of IAPs through an increase in CRAF and Rac1 levels (Fig. 3). Indeed dual targeting of IAPs and Rac1 effector PAK1 has been shown to be effective in inducing effector caspase activation and apoptosis in NSCLC tumor cells.⁴⁶ A better understanding of the role of IAPs in regulating tumor cell migration and invasion will assist in patient selection as well as to adroitly administer IAP antagonist compounds in treating a complex disease like cancer. The discovery of IAPs and HACE1 as the E3 ubiquitin ligases of Rac1 has revealed the molecular mechanisms behind polyubiquitination of Rac1, a thus far uncharacterized layer of regulation apart from GEFs, GAPs and RhoGDIs.

Acknowledgements

We would like to thank all members of K.R. lab and our collaborators for insightful discussions. The work discussed here is primarily funded through an ENP program grant RA1739/1-1 from the D.F.G. to K.R.

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